UNIVERSITY OF CALIFORNIA SAN DIEGO

Serotonergic plasticity in the dorsal raphe nucleus characterizes susceptibility and resilience to anhedonia

A dissertation submitted in partial satisfaction of the requirements for the degree

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in

Biology

by

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The Dissertation of Nandkishore Prakash is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

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2019

DEDICATION

To the Truth

EPIGRAPH

"*...Never mind the struggles, the mistakes…*

...these failures, these little backslidings; hold the ideal a thousand times

and if you fail a thousand times, make the attempt once more…"

Swami Vivekananda

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LIST OF ABBREVIATIONS

30G	Thirty Gauge
µA	microampere
µl	microliter
µm	micrometer
AAALAC	American association for accreditation of laboratory animal care
AAVDJ	Adeno-associated virus, serotype DJ
ANOVA	Analysis of variance
AP	Antero-posterior stereotaxic coordinate
Avg	Average
***Cre* / Cre**	Gene or protein (respectively) corresponding to Cre-recombinase
DIO	Double floxed inverse orientation
DNA	De-oxyribonucleic acid
DREADD	Designer receptor exclusively activated by designer drug
DV	Dorso-ventral stereotaxic coordinate
EDTA	Ethylenediaminetetraacetic acid
Fev	Fifth Ewing variant. An alias for *Pet1*. See *Pet1*
eGFP	enhanced Green fluorescent protein

g / kg	grams per kilogram
GABA	Gamma-aminobutyric acid
GC / mL	Genome copies per milliliter
hM3Dq	Excitatory DREADD receptor
HPA	Hypothalamic pituitary axis
HSD	Honestly significant difference (Tukey's test)
hSyn	human synapsin promoter
Hz	Hertz
IM	Intra-muscular
IP or i.p.	Intra-peritoneal
mg / kg	milligrams per kilogram
miRNA	micro RNA (Ribonucleic acid)
ML	Medio-lateral stereotaxic coordinate
mm	millimeter
mM	millimolar
mRNA	messenger RNA (Ribonucleic acid)
NaCl	Sodium chloride
NaOH	Sodium hydroxide

Nurr1	Nuclear receptor related 1
PCR	Polymerase chain reaction
pH	power of hydrogen
***Pet1* / PET1**	mRNA or protein (respectively) for plasmacytoma expressed transcript 1
s.e.m.	standard error of mean
Syn1	Synapsin 1 promoter
***Tph2* / TPH2**	mRNA or protein (respectively) for tryptophan hydroxylase 2
TPH2+ / TPH2-	Immunopositive or immunonegative for TPH2 protein
***Tph2*+ / *Tph2*-**	Positive or negative for carrying *Tph2* mRNA transcript
Tris HCl	Tris (hydroxymethyl) aminomethane hydrochloride
v / v	volume by volume
***Vglut3* / VGLUT3**	mRNA or protein (respectively) for vesicular glutamate transporter 3

LIST OF SYMBOLS

α	read as ‘anti’. Denotes that antibody was raised ‘against’ a specific target antigen or species e.g. Rabbit α TPH2 indicates antibody from a rabbit host ‘against’ the target antigen TPH2. Donkey α Sheep denotes a secondary antibody raised in a donkey host against primary antibodies raised in sheep.
χ^2	read as ‘chi-squared’. Test statistic for Kruskal-Wallis H test
ε	read as ‘epsilon’. Denotes degree of sphericity
F	Test statistic for ANOVA
n	sample size for a given experimental group in a given experiment
p	read as ‘p-value’. Probability that the test statistic comes from the null distribution
r (Pearson’s r)	bivariate Pearson’s correlation coefficient
±	read as ‘plus or minus’. Denotes symmetric range around an observation
+/-	Indicates that error bars are plotted either for positive or negative error value but apply equally in both directions
3’	Denotes 3 ‘minutes’
60’’	Denotes 60 ‘seconds’

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ACKNOWLEDGEMENTS

An entire lifetime appears to have passed in the six years that I spent at UCSD; six years of stupendous personal and professional growth that culminates in the writing of this dissertation, and stepping out into the world as a scientist equipped with not just a skillset or a mindset, but with an experiential wisdom borne from an arduous, but highly instructive time as a graduate student. Six years with the punch of sixty.

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I reserve the best for the last – my friends and family. Indeed, a magical experience for me in San Diego has been the comfort of family-like love from my closest friends, and the staunch

friendship provided by my family; a very precious addition to the typical companionship of friends and love from family. First, my deep respect and admiration for Siddhartha Nath, a friend and *guru*. Making his acquaintance, brought back beautiful music in a lasting way, an immense contribution to my life that cannot be overstated or fully reciprocated. It is also thanks to him that I found and developed invaluable friendships with so many others. Next, my first experience of a friend who cared as much as family did – Swetha Godavarthi. Her contribution to my success as a PhD candidate is staggering and very difficult to describe. It suffices to say that without her part in this, I would have given up a long time ago. In addition, she introduced to me to the joy of dancing, a newfound interest that I will carry forward. My love, joy and gratitude know no bounds when it comes to my closest friends Vignesh, Akshat, Sourish and Pritesh. It is quite astonishing that within three to five years these remarkable people, whom I count as my rocks and my inspiration, became as close to me as my family. The music, laughter and long hours in their company have kept me going ahead with energy and eagerness. Each of them has a unique, lasting and unforgettable contribution to my journey. Special thanks to Aaron Sampson for being such a cool roommate who taught me so much about the States, rekindled my gardening interest, put up with my singing and was always around to lend a helping hand. I've had the most amazing circle of friends with whom I've shared dinners, hikes, music, parties, festivals, games, cooking, laughter and so much more. The list may be long but they all deserve a special mention: Abhay, Adithya, Aekaansh, Amol, Animesh, Annie, Bhanu, Bharat, Kanika, Mihir, Mrittika, Neeraja, Raghu, Suhas, Sumitash, and last but certainly not the least, Sunandha and Tapan. My fellow Biology PhD classmates, Ami, Angela, Ipshita, Sachin, Maria, Will and the others also gave me a really memorable and fun time, while being greatly supportive.

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Research in experimental neuroscience using animal models implies the contribution of scores of little, innocent lives that unfortunately have to be sacrificed in the human pursuit of answers and cures. While I cannot meaningfully thank them, I now take a moment to remember them as the silent heroes of this story – the many rats whose lives and deaths were dedicated in entirety, without their choice, towards the human endeavor that is science.

Chapter 2, in full, has been submitted for publication of the material as it may appear in Prakash N, Stark CJ, Keisler MN, Luo L, Der-Avakian A, Dulcis D (2019) Serotonergic plasticity in the dorsal raphe nucleus characterizes susceptibility and resilience to anhedonia. The dissertation author was the primary researcher and author of this paper.

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ABSTRACT OF THE DISSERTATION

Serotonergic plasticity in the dorsal raphe nucleus characterizes susceptibility and resilience to anhedonia

by

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Doctor of Philosophy in Biology

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Professor Davide Dulcis, Chair

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Major depressive disorder (MDD) and other depressive disorders are highly prevalent in the world today. A pervasive and debilitating symptom of MDD, that is also seen in other depressive disorders, is anhedonia – the lack of interest or pleasure, a consequence of dysfunction

of reward circuits. Chronic stress induces anhedonia in susceptible, but not resilient individuals, a phenomenon observed in humans as well as animal models. The molecular mechanisms underlying this are not well understood. The study described in this dissertation was based on the hypothesis that plasticity of the serotonergic system, which is implicated in stress, reward and antidepressant therapy, plays a role in determining susceptibility and resilience. Stress-induced anhedonia was assessed in adult male rats using social defeat and intracranial self-stimulation (ICSS), while changes in serotonergic phenotype were investigated using immunohistochemistry and *in situ* hybridization. Susceptible, but not resilient, rats displayed an increased number of neurons expressing the enzyme for serotonin, tryptophan-hydroxylase-2 (TPH2), in the ventral subnucleus of the dorsal raphe nucleus (DRv). Further, a decrease in the number of DRv neurons expressing the glutamatergic marker, vesicular-glutamate-transporter-3 (VGLUT3), was observed in all stressed rats. Neuronal activity was decreased in the DRv, specifically in non-serotonergic neurons, in all stressed rats. These changes occurred without a change in total neuron number in the DRv, suggestive of a neurotransmitter plasticity mechanism. This was dependent on DR activity, as was revealed by chemogenetic manipulation of the central amygdala, a stress-sensitive nucleus that forms a major input to the DR. Activation of amygdalar corticotropin releasing hormone (CRH)+ neurons abolished the increase in DRv TPH2+ neurons and ameliorated stress-induced anhedonia in susceptible rats. Therefore, activation of amygdalar CRH+ neurons induces resilience, and suppresses the characteristic phenotype of susceptible rats. The molecular signature of vulnerability to stress-induced anhedonia and the active nature of resilience could be a target of new treatments for stress-related disorders like depression. This dissertation also discusses further research questions that can be pursued in the light of this study and the experimental approaches to address them.

CHAPTER 1

The biology of mental health – An introduction

1.1 Mental health disorders and why they matter

The preamble to the constitution of the World Health Organization[1] defines health as "a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity"; thus, recognizing mental well-being as an integral component of human health. The WHO further describes *mental health* as "a state of well-being in which the individual realizes his or her own abilities, can cope with the normal stresses of life, can work productively and fruitfully, and is able to make a contribution to his or her community"[2]. It follows then, that disturbances in mental well-being would affect normal functioning, reduce an individual's contribution to society and put him or her at a socio-economic disadvantage. Therefore, the research, diagnosis and treatment of mental health conditions is an enterprise of utmost importance to an equal, productive and healthy society.

Unfortunately, epidemiological studies have painted a grim picture of the prevalence of mental disorders in the world today. The burden on a population, attributable to a particular disease, a class of diseases, or all diseases, is quantified by epidemiologists using several metrics. Disability-adjusted life years (DALY) is one such metric, which conveys the number of years of healthy life *lost* to death or impairment[3]. It factors in the number of years lost to death and the number of years lived with a disability due to a particular cause, calculated relative to a goal of living in full health up to standard life expectancy. A higher DALY figure reflects a poorer state of health or a larger gap (deficit) between the current state and the standard goal.

In 2010, mental disorders accounted for 7.4% of the global DALYs, marking a 37.6% increase from the year 1990[4]. More recent data indicate a further 13.5% increase between the years 2007 and 2017[5]. It has even been argued that some of these data may have been under-estimated[6]. These metrics alone convey the high prevalence of mental and behavioral disorders and an urgent need to tackle them systematically.

The term 'mental and behavioral disorders' describes a fairly heterogeneous group of conditions. Psychiatry, the branch of medicine that focuses on mental, emotional and behavioral disorders, has been evolving in thought and practice over the last century; and this has led, among other developments, to a more detailed and systematic classification of these disorders and their diagnosis[7]. Categorizing mental disorders and establishing clear and precise diagnostic criteria are critical to (i) providing effective treatment to patients, (ii) understanding the epidemiology of each disorder and (iii) conducting research in humans or animal subjects for specific disorders or symptoms. The Diagnostic and Statistical Manual of Mental Disorders (DSM-5)[7] broadly categorizes mental disorders into multiple categories including, depressive disorders, schizophrenia spectrum and other psychotic disorders, anxiety disorders, bipolar and related disorders, substance-related and addictive disorders, among others.

Depressive disorders led other mental disorders in contribution to global burden, by accounting for 40% of the DALYs within the mental and behavioral disorders category, as revealed by the Global Burden of Disease Study 2010[8] and continued to lead nearly a decade later by accounting for 35% of DALYs contributed by mental disorders[5]. This was seen across all age groups, starting from the age of 5 and across geographical regions[8]. Moreover, major depressive disorder or MDD, a classic representative of depressive disorders, was ranked the 11th most common disease contributing to global burden in 2010, up from the 15th position it was placed at

in 1990[4]. Sex differences are observed in the incidence of depressive disorders. Within females, depressive disorders, which ranked at #17 in 1990, went up to #11 in 2017; and from #30 to #23 within males during the same period[5]. Analysis of another metric of disease burden, called 'years lived with disability' or YLD, put depressive disorders among the top 3 contributors to global YLDs in 2017 for both sexes combined[9]. YLD is a component of DALY that only accounts for years lived with an impairment and excludes years lost to premature death[10].

These data imply that MDD and other depressive disorders constitute a major threat to health and are the leading cause of global burden of mental and behavioral dysfunction. This justifies the need to have a thorough understanding of the pathophysiology and treatment of MDD and other depressive disorders.

1.2 Major depressive disorder – diagnosis, treatment and the need for research

MDD is characterized by episodes including the core symptoms of (i) depressed mood and feelings of sadness, and/or (ii) *anhedonia*, the lack of interest or pleasure in nearly all activities; nearly every day, for most of the day within a two-week period. An episode of MDD comprises at least five symptoms (including at least one of the above), the others being drastic changes in appetite or weight, sleep, psychomotor behavior, energy level, impaired memory or decision-making, feelings of worthlessness and recurrent thoughts of death or suicide. When these symptoms manifest to an extent where normal functioning (social, at work etc.) is significantly impaired, and cannot be attributed to the use of a substance or to another medical condition, the patient is diagnosed with MDD[7].

The first lines of treatment for MDD typically include psychotherapy and various antidepressant medications in different combinations and strengths[11]. About 10% - 30% of MDD-

affected individuals[12] do not respond to these treatments and are therefore diagnosed as having treatment-resistant depression[13]. Treatment-resistant depression needs more complex, expensive and/or invasive approaches to treatment such as electroconvulsive therapy (ECT), transcranial magnetic stimulation (TMS), transcranial direct current stimulation (tDCS) and deep brain stimulation (DBS)[14,15].

Although many treatment approaches for MDD have been in clinical practice for decades, little is known about their neurobiological bases. This lack of mechanistic knowledge is due in part, to the fact that many of these discoveries were made serendipitously[14] or driven by looking for clinical outcomes[15], rather than on the basis of fundamental biological principles. It is also due to the historical lack of tools for high-resolution genetic, molecular, anatomical, electrophysiological and behavioral investigations in humans and animals. Recent advances in neurobiological tools like optogenetics, chemogenetics, viral tracers, multi-electrode local field potential (LFP) recordings, patch-clamp recordings, electroencephalography (EEG), *ex vivo* and *in vivo* calcium imaging, caged neurotransmitter release and animal behavior testing, combined with advances in other areas of biology, chemistry and data analytics have vastly improved our ability to understand biological details of psychiatric phenomena. The use of animal models of human behavior has been a key advance in biological psychiatry, a prime example being the use of stress to induce depression-like behavior in animals[16–18].

Stress-induced depressive behavior in animals has translational relevance because stress can lead to MDD in vulnerable individuals without prior history of psychiatric disorders[19–21]. The effects of stress include, but are not limited to, anhedonia[20,21]. Anhedonia is a symptom of particular interest, not only because it is one of the two core symptoms of MDD, but also because it can be experimentally evaluated in animals unlike ‘depressed mood’ or ‘feelings of sadness’. It

is also seen in other disorders such as bipolar disorders[7]. Animal models of stress-induced anhedonia[22] have been developed based on a combination of their reliability (whether effects in the model are repeatable and reproducible), etiological and predictive validity (whether causes and effects in the model parallel those in humans), face validity (how well the model recapitulates various aspects of the human condition), construct validity (accuracy of measurement in the model based on a theoretical understanding of the condition being modeled) and convergent and discriminant validity (how well the model agrees with or differs from other models)[23,24]. With constant refinement based on these criteria, animal models of stress-induced anhedonia have proven to be useful for investigations into the neurobiology of MDD and other stress-induced disorders.

1.3 Animal models of stress-induced anhedonia and depression

1.3.1 Primate models of stress-induced depression

Non-human primates (NHPs) are evolutionarily the closest species to humans among laboratory animals, and demonstrate a range of normal and pathological behaviors that mimic those of humans[25]. This has prompted researchers to use them as animal models using various stressors to induce depression-like behavior. Chronic isolation stress in common marmosets[26], manipulation of social status in cynomolgus monkeys[27] and maternal separation in pigtail, bonnet and rhesus macaques[28–30] are examples of good NHP models of human depression. However, the availability and sophistication of tools to alter gene expression, and to record and manipulate neuronal activity with cellular resolution, are more developed in rodents; making them better suited for finer mechanistic studies.

1.3.2 Rodent models of stress-induced depression

Mice and rats are the most studied laboratory models of stress-induced depression owing to the ease of their rearing, the availability of tools and the diversity in their behavioral repertoire. The characteristics of the stressor used and the behavioral readout of the depressive state vary across individual models, and influence their interpretation and translational relevance.

Acute stressors such as single-episode social defeat, foot-shock, acute cold swim stress, tail suspension and acute immobilization stress are typically of short duration (up to 1-2 hours for a single day) and are used to study immediate neuroendocrine and behavioral effects of stress[31–34] or their impact on vulnerability to future stressors[35]. Chronic stressors such as early maternal separation, chronic social defeat, chronic unpredictable mild stress, chronic restraint and chronic isolation typically extend for at least 10 days, and are used to study depression or post-traumatic stress disorder[16,36–40]. In addition, pharmacological agents like corticosterone[41] or LPS[42,43] are used to induce stress and model stress-induced depression.

The study described in this dissertation utilized chronic social defeat as a stressor (see section 2.4.5 for details of the protocol). The choice of stressor was motivated by several criteria: (i) ethological relevance – social defeat is a display of social and territorial aggression / submission that is a part of the natural behavioral repertoire of rodents unlike foot-shock or restraint; (ii) strength – it is a strong stressor as evidenced by the multiple effects of acute and chronic defeat[44–46] and by the fact that only chronic (but not acute) treatment with antidepressants can reverse its effects[47], (iii) reliability – social defeat produces consistent, reproducible effects within subjects and across laboratories[48] and (iv) validity – predictive, face and construct validity for depression[49].

As mentioned above, the behavioral readout (assay) of depression is also an important aspect of the model. Various behaviors have been measured to assess the extent of depression such as, reward consumption, locomotor activity, forced swimming, social interaction, etc.[50–52]. Tests that measure reward consumption (considered to be a readout of anhedonia) are preferred over others since tests of anhedonia have better specificity and face validity for depression[53]. Sucrose consumption / preference, intracranial self-stimulation (ICSS), reward-induced place preference conditioning are common assessments of anhedonia. Of these, only ICSS provides a reliable, continuous, quantitative measure of anhedonia that is not confounded by taste or metabolic factors. These reasons motivated the use of ICSS in the study described in Chapter 2. Methodological details of ICSS are provided briefly in section 2.4.4 and in greater detail by[54]. ICSS encompasses consummatory and motivational aspects of reward, meaning that the ability to experience reward and the motivation to obtain it, both play a role in a rodent's performance on the ICSS task; and therefore, changes in ICSS behavior can be attributable to changes in either of these two aspects of reward processing. This is in contrast to tests such as progressive ratio breakpoint (specific to motivation), probabilistic reversal learning (specific to learning and decision-making aspects of reward response), or positive-negative contrast for sucrose preference (specific to anticipation of reward)[50].

1.4 The complexity of depression: revelations by animal models

A multitude of brain regions have been implicated in depression, such as the prefrontal cortex, hippocampus, amygdala, lateral hypothalamus, habenula, bed nucleus of stria terminalis and the raphe nuclei[55–58]. Dysregulation of normal physiology in these regions, which can be induced by stress, gives rise to the symptoms of depression. This involves a variety of mechanisms at the genetic[59], epigenetic[60], molecular[61], cellular[62–64], synaptic[65], physiological[66,67] and circuit[68,69]

levels. Since the late 2000s, a prominent focus of depression research has been to understand individual differences in response to stress, and the mechanisms behind susceptibility and resilience to stress-induced depression in animal models[51,70–73]. Many biomarkers of susceptibility and resilience have since been discovered[74,75]. This provides critical insight into the neurobiology of human vulnerability and resilience to stress[19,76,77].

Genetic studies in humans and animals have demonstrated a link between the brain's serotonergic system and vulnerability to stress-induced depression[78,79]. But there is little detail on the role of the serotonergic system in determining susceptibility or resilience, which is surprising as it has otherwise been studied heavily in the context of depression and antidepressants[80–82]. Barring a few exceptions[38,83,84], most studies on the serotonergic system do not differentiate between susceptible and resilient individuals. This lack of clarity prompted the investigation described in Chapter 2. The following section provides a brief introduction to the serotonergic system.

1.5 Serotonin in the brain

'Serotonergic system' is a broad term that refers to the molecules, neurons and brain regions that are involved in signaling using the neuromodulator serotonin. Serotonin, or 5-hydroxy tryptamine (5-HT), is a derivative of the amino acid tryptophan and is synthesized in the central nervous system exclusively by the enzyme tryptophan hydroxylase isoform 2 (TPH2)[85,86]. Extracellular serotonin in the synapse is either taken back into the pre-synaptic compartment by serotonin transporters (called serotonin reuptake) or degraded by monoamine oxidase[87,88]. Serotonergic neurons are characterized by the expression of transcription factor genes such as *Lmx1b* and *Pet1*, and other molecules such as SERT, TPH2, VMAT, MAO and 5-HT itself[89].

The raphe nuclei, located in the brainstem, contain most of the cell bodies making serotonin in rodent as well as human brains[90,91]. They project to diverse cortical, subcortical and spinal targets[91,92] where they exert excitatory or inhibitory effects via a panoply of 5-HT receptors[93]. The raphe nuclei are broadly divided into two groups – rostral and caudal. The rostral group contains most of the serotonergic cell bodies, of which a majority are located in the dorsal raphe nucleus (DR)[91,94,95]. The DR receives afferents from regions such as the central amygdala, bed nucleus of stria terminalis, locus coeruleus, habenula, orbitofrontal cortex and ventral tegmental area. Efferents from the DR reciprocally innervate most of these same regions[96]. This connectivity enables the DR to acts as a hub for processing stress, reward and emotion-based decision making.

In rodents, the DR is further subdivided into six subnuclei – mesencephalic, dorsal, ventral, ventrolateral, interfascicular and caudal. The patterns of connectivity and neurotransmitter expression differ across subnuclei[97], making the DR a very heterogeneous nucleus with multiple functions including regulation of mood, sleep, reward and decision-making[98–100].

The complex interactions between stress, antidepressants and the serotonergic system affect the expression of several molecules[101], firing rates of different neuronal types and extracellular 5-HT levels[81]. An interesting finding in this regard is that depressed human patients who committed suicide had an increased number of TPH2 immunoreactive neurons in the DR[102]. Since TPH2 expression identifies serotonergic neurons, an increase in the number of TPH2+ neurons, implies an increase in serotonergic neurons. This is of particular significance because the increase in TPH2 expression is not simply at the level of mRNA or protein within neurons that were already serotonergic, but within another population of neurons that gains TPH2 expression. In a nucleus such as the DR, which is not known to display adult neurogenesis, the increase in TPH2-immunoreactive neurons must therefore be a result of previously non-serotonergic neurons

gaining TPH2. This appears to be an example of a form of plasticity called neurotransmitter plasticity[103] which is described in the next section.

1.6 Neurotransmitter plasticity

In the early days of cellular neuroscience, it was believed that a neuron releases only one neurotransmitter throughout its lifetime, and that the identity of this neurotransmitter is fixed at the time of its birth[104]. This principle was referred to as Dale's principle and while the documentation of several exceptions has disproved it, the notion of 'neurotransmitter identity' of a neuron has endured; and terms such as 'serotonergic', 'glutamatergic' or 'GABA-ergic' are still in common use oftentimes with the assumption that a neuron uniquely expresses one transmitter.

Early cell culture studies showed that neurotransmitter phenotype is plastic[105,106]. This was later also demonstrated *in-vivo* and the term 'neurotransmitter plasticity' was first used in 1983 in the context of developing sympathetic innervation of sweat glands[107]. Briefly, neurotransmitter plasticity is the loss or gain of a neurotransmitter by a population of neurons, presumably occurring at the single-cell level in a subset of the population. When this loss or gain of neurotransmitter is accompanied by a gain or loss, respectively, of another transmitter, it is called neurotransmitter switching. The neurons undergoing this plasticity are mature, differentiated neurons that are already a part of pre-existing circuits and form a 'reserve pool' for neurotransmitter switching[108].

The neurotransmitter phenotype of neurons is activity-dependent and can be manipulated *in vitro* and *in vivo*. Altering population activity by gene overexpression or by imposing calcium transients on neurons could direct their neurotransmitter phenotype[109,110]. Further, it was demonstrated that activity-dependent transmitter plasticity *in vivo* is accompanied by a corresponding change in postsynaptic receptors[111]. Taken together these findings suggest that

neurotransmitter plasticity is homeostatic, i.e. it occurs as a corrective mechanism to maintain the activity of a neuronal population at its natural set-point; presumably to prevent runaway changes in neural activity that may have adverse consequences on an animal's behavior. Guemez-Gamboa et al.[112] found that neurotransmitter plasticity is non-cell autonomous, meaning that within a neuronal population of which some neurons are directly experiencing a change in activity via their synaptic inputs, even other neurons that are not directly affected can alter their neurotransmitter expression to homeostatically regulate the overall level of excitation in the population. These early studies utilized the developing amphibian spinal cord or neuromuscular junction to explore neurotransmitter plasticity, but the relevance of this phenomenon to the animal's ethology was not clear.

Later, environmentally-induced neurotransmitter plasticity in the developing central nervous system that impacted the animal's survival ability was discovered[113]. The first demonstration of neurotransmitter plasticity in the adult mammalian brain was by Dulcis et al.[116] who found that altering photoperiod exposure can induce a dopamine-somatostatin switch in the para- and peri-ventricular nuclei of the hypothalamus and consequently regulate anxiety-like behavior in rodents. Subsequently, neurotransmitter plasticity has been observed in animal models of drug addiction, bipolar disorder and HIV-infection[115–117].

1.7 Hypothesis and rationale for the dissertation study

Until the study described in Chapter 2 was undertaken, the idea of neurotransmitter plasticity as a candidate phenomenon to explain susceptibility and resilience to stress-induced anhedonia (depression) had not been tested. However, four major considerations favored this idea:

(i) The chronic nature of the stressor (chronic social defeat), and the time period over which susceptibility and resilience manifest in response to stress[38] align with the timeline of neurotransmitter plasticity as understood from previous studies[114].

(ii) The past literature detailing the role of serotonin in stress, depression and anti-depressant action also backs the idea that a form of neural plasticity involving neurotransmitter action may play a role in susceptibility and resilience.

(iii) The subnucleus-specific expression and overlap of multiple neurotransmitters in the DR, indicate that the DR has a heterogeneous molecular character that makes it a fertile ground for investigation of neurotransmitter plasticity.

(iv) Finally, the findings by Underwood et al.[102], mentioned in Section 1.5, are the strongest hint that neurotransmitter plasticity involving serotonin plays a role in the pathophysiology of depression.

These considerations led to the formulation of the hypothesis. For reasons described in Sections 1.2 and 1.3.2, anhedonic behavior was used as the readout of depressive state.

Hypothesis – '*Plasticity of serotonergic expression in the DR plays a role in susceptibility and resilience to chronic stress-induced anhedonia.*'

1.8 Outline of the dissertation

In summary, MDD and other depressive disorders are a major public health concern in the world today. Research in animal models of depression yield valuable insights into the neurobiology of depression which can be used to design and understand treatment approaches. In particular, rodent models of chronic stress-induced anhedonia (a classic hallmark of depression) point to the role of the brain's serotonergic system in the pathophysiology of depression and

mechanism of antidepressant action. While susceptibility and resilience to stress-induced anhedonia has been observed in animals and humans, the role of the serotonergic system in this context had not been explored. Available literature suggested that neurotransmitter plasticity in the serotonergic system may play a role but this idea had not been tested. These considerations prompted the formulation of the hypothesis stated in Section 1.7.

Chapter 2 details the materials and methods used to test the hypothesis, the results obtained and conclusions derived, following a brief abstract and introduction. Chapter 3 then discusses new questions raised by the results described in Chapter 2 and the experiments that can be performed to answer them.

CHAPTER 2

Serotonergic plasticity in the dorsal raphe nucleus characterizes susceptibility and resilience to anhedonia

2.1 Abstract

Chronic stress induces anhedonia in susceptible, but not resilient individuals, a phenomenon observed in humans as well as animal models, but the molecular mechanisms underlying susceptibility and resilience are not well understood. We hypothesized that the serotonergic system, which is implicated in stress, reward and antidepressant therapy, may play a role. We found that plasticity of the serotonergic system contributes to the differential vulnerability to stress displayed by susceptible and resilient animals. Stress-induced anhedonia was assessed in adult male rats using social defeat and intracranial self-stimulation (ICSS), while changes in serotonergic phenotype were investigated using immunohistochemistry and *in situ* hybridization. Susceptible, but not resilient, rats displayed an increased number of neurons expressing the biosynthetic enzyme for serotonin, tryptophan-hydroxylase-2 (TPH2), in the ventral subnucleus of the dorsal raphe nucleus (DRv). Further, a decrease in the number of DRv glutamatergic neurons was observed in all stressed animals. This neurotransmitter plasticity is dependent on DR activity, as was revealed by chemogenetic manipulation of the central amygdala, a stress-sensitive nucleus that forms a major input to the DR. Activation of amygdalar corticotropin releasing hormone (CRH)+ neurons abolished the increase in DRv TPH2+ neurons and ameliorated stress-induced anhedonia in susceptible animals. These findings show that activation of amygdalar projections induces resilience, and suppresses the gain of serotonergic phenotype in the DR that is characteristic of susceptible animals. This molecular signature of vulnerability to stress-induced

anhedonia and the active nature of resilience could be a target of new treatments for stress-related disorders like depression.

2.2 Significance

Depression and other mental disorders can be induced by chronic or traumatic stressors. However, some individuals are resilient and do not develop depression in response to chronic stress. A complete picture of the molecular differences between susceptible and resilient individuals is necessary to understand how plasticity of limbic circuits is associated with the pathophysiology of stress-related disorders. Using a rodent model, our study identifies a novel molecular marker of susceptibility to stress-induced anhedonia, a core symptom of depression, and a means to modulate it. These findings will guide further investigation into cellular and circuit mechanisms of resilience, and the development of new treatments for depression.

2.3 Introduction

Anhedonia, or lack of interest or pleasure, is a debilitating symptom of several psychiatric and neurological disorders, including major depressive disorder (MDD)[7] that reflects impaired brain reward function. It has been modeled in rodents and primates using multiple behavioral paradigms that capture different aspects of reward processing[50,118,119].

The intracranial self-stimulation (ICSS) procedure provides a direct and quantitative measure of anhedonia in rodents[120–122], which can be induced by stressors like social defeat[18,38,123,124]. However, not all stressed animals develop anhedonia[38,51], a phenomenon mimicking resilience to stressful or traumatic experiences in humans[19,125]. The mechanisms determining susceptibility or resilience to stress-induced anhedonia have important implications for understanding and developing treatments for disorders like MDD[126].

Serotonergic transmission in the brain is implicated in the processing of stress[127–129], reward[130–134] and emotional behaviors[135–137]. Its role in the pathophysiology of many neurological[138,139] and psychiatric disorders[140–142] is well established. Knockout mice lacking key components of serotonergic machinery show altered stress-related behaviors[143–146]. Extracellular levels of serotonin[147–149], expression of serotonin-related molecules[150–153], serotonergic activity[154–156] and innervation[157] have been shown to change in response to both acute and chronic stress. Such regulation is linked to behavioral changes including anhedonia[157–160] and is altered by treatment with antidepressants and anxiolytics[161,162]. Accordingly, we investigated serotonergic circuitry for mechanisms that could explain susceptibility and resilience to stress-induced anhedonia.

Serotonin (5-hydroxytryptamine or 5-HT) is mainly synthesized in the raphe nuclei[90,91,94,163] by the enzyme tryptophan hydroxylase 2 (TPH2)[85,86]. Among the raphe nuclei, the dorsal raphe nucleus (DR) accounts for most serotonergic cell bodies both in rat[91] and human brains[95,164]. Furthermore, serotonergic transmission occurs at multiple sites in the central nervous system[90–92].

Earlier studies have found that the number of serotonergic neurons[102], levels of TPH2 mRNA[165] and TPH2 protein[166] are elevated in the DR of deceased patients who committed suicide. These results led us to hypothesize that chronic stress-induced plasticity of serotonergic expression[167] may occur in the DR and play a role in determining susceptibility or resilience to anhedonia. Neurotransmitter plasticity[108,168] has not been explored previously in the DR or other brain regions in animal models of anhedonia.

Here, we describe a mechanism of transmitter plasticity involving increased number of TPH2+ neurons in the DR in susceptible rats following chronic stress. We further demonstrate that manipulation of DR activity can rescue the transmitter phenotype and behavior.

2.4 Materials and Methods

2.4.1. Animals

Adult male rats were used for all experiments. All rats were housed in a 12h reverse light-dark cycle with *ad libitum* access to food and water. Wistar rats (Charles River Laboratories, Kingston, NY) weighing 300-400g (~8 weeks old) were used for ICSS electrode implantation surgeries, behavior and immunohistochemistry, in experiments without chemogenetic manipulation. *Crh-Cre* transgenic male Wistar rats (rat line generously provided by Dr. Robert O. Messing, University of Texas at Austin) were bred in our vivarium and used for the chemogenetics experiment. Details of development of the *Crh-Cre* rats are described by Pomrenze et al.[169]. For social defeat, male Long-Evans rats (retired breeders; Charles River Laboratories) co-housed with females and litters were used as resident aggressors. All rats were pair-housed except during social defeat. *Crh-Cre* breeding pairs were at least 10 weeks old and either pair-housed or harem-housed (two females with one male). Pups were weaned from the dam and genotyped 21 days after birth. All experiments were carried out in accordance with the guidelines of AAALAC International and National Research Council's Guide for the Care and Use of Laboratory Animals and approved by the UCSD Institutional Animal Care and Use Committee.

2.4.2 Genotyping

Ear tissue punches (2 mm diameter) were collected from *Crh-Cre* progeny for DNA extraction and genotyping. For DNA extraction, tissue was incubated in 75 µl alkaline lysis buffer

(25 mM NaOH, 0.2 mM EDTA, pH 12.0) at 95°C for 1h followed by addition of equal volume of neutralization buffer (40 mM Tris-HCl, pH 5.0) and short-term storage at 4°C. The mixture was used as source DNA for PCR-based genotyping. PCR protocol: 0.5 µl of DNA, 0.5 µl each of forward and reverse primers, 5 µl of KAPA2G Fast HotStart ReadyMix (KK5603, KAPA Biosystems) and 3.5 µl of sterile water were mixed in a 10 µl reaction. Cre recombinase forward primer, 5'-GCATTACCGGTCGATGCAACGAGTGATGAG-3'
and reverse primer, 5'-GAGTGAACGAACCTGGTCGAAATCAGTGCG-3' (Washington University Mouse Genetic Core, mgc.wustl.edu)
were used. Cycling parameters were 95°C for 3'; 30 cycles of 95°C for 15'', 60°C for 60'', 72°C for 40''; 72°C for 2' in a T100™ Thermal Cycler (Bio-Rad) followed by long-term storage at -20°C. PCR product was analyzed by horizontal agarose gel electrophoresis and presence of 550 bp band was determined in order to identify Cre-positive progeny.

2.4.3 Surgery

For ICSS electrode implantations, rats were anesthetized with a 5% isoflurane/oxygen vapor mixture and attached to a stereotaxic frame (Kopf Instruments; Tujunga, CA) where continuous flow of 2% isoflurane/oxygen was administered throughout the procedure. The incisor bar was set at 5.0 mm above the interaural line. Bipolar insulated stainless-steel electrodes (11 mm length, model MS303/2; Plastics One; Roanoke, VA) were unilaterally (counterbalanced) implanted in the posterior lateral hypothalamus (AP -0.5 mm, ML ± 1.7 mm from bregma and DV -8.3 mm from dura). The electrode was secured using dental acrylic and 4-6 stainless steel jeweler's screws. The exposed electrode pedestal was shielded using a metal screw cap to prevent damage.

For chemogenetics, bilateral viral injections (1.0 µl/side) into the lateral subnucleus of the central amygdala (AP -2.3 mm, ML ± 4.7 mm from bregma and DV -6.9 mm from dura, head parallel to horizontal) were performed using a 30G metal cannula (PlasticsOne) connected to a Hamilton syringe pump (10 µl syringe) at a rate of 0.1 µl/min prior to electrode implantation during the same surgical procedure. Rats were injected with either the control virus (AAVDJ-Syn1-DIO-eGFP, 1.78E+13 GC/mL, Salk Institute; La Jolla, CA) or the excitatory DREADD receptor-encoding virus (AAV5-hSyn-DIO-hM3Dq-mCherry, 6.50E+12 GC/mL, Addgene, Watertown, MA). Viral incubation occurred for at least 8 weeks during the post-surgical recovery, ICSS training, baseline testing and saline habituation periods. Post-surgical treatment with topical antibiotic cream and 20 mg/kg of enrofloxacin IM was provided to prevent infection.

2.4.4 ICSS apparatus, training, testing and analysis

The ICSS procedure, including apparatus, training and testing, was performed as previously described[37]. Briefly, rats were trained to seek reinforcement of direct current stimulation of the posterior lateral hypothalamus by turning a wheel manipulandum in the testing chamber. A non-contingent stimulus (100 Hz electrical pulse train) of current intensity varying from 50 to 300 µA was delivered to the rat by means of a computer-controlled constant current stimulator (Stimtek Model 1200c; San Diego Instruments, San Diego, CA). Rats were trained to turn a wheel in response to the non-contingent stimulus to receive a second (contingent) stimulus with identical parameters. Current intensities were systematically varied across trials, separated by inter-trial intervals without stimulation. Using this discrete-trial current-intensity procedure, the minimum current intensity required to elicit a response from the rat was measured and defined as the reward threshold.

Reward thresholds were measured every day, at the same time, in trained rats. Thresholds were monitored for 3 to 6 weeks until they were stable (i.e., <10% variation over 5 consecutive days). The baseline threshold for each rat was calculated as the average of its daily thresholds for 3 days prior to the beginning of testing. Rats with stable thresholds were divided into control and stress groups. For the chemogenetics experiment, litter effects were avoided by distributing rats from different litters across groups. Controls were tested in the ICSS procedure daily and stressed rats were tested daily within 15 minutes of a social defeat encounter.

Elevations in thresholds indicated that a greater current intensity was required to generate positive reinforcement, reflecting an anhedonic state. A rat was classified as susceptible to anhedonia if its average threshold during days 19-21 of social defeat was greater than 3 standard deviations from the pre-defeat baseline thresholds of the cohort (calculated by averaging baseline thresholds across all stressed rats). Daily reward thresholds were plotted as percent changes from baseline averaged across rats in each group.

2.4.5 Chronic social defeat

A resident-intruder procedure was used as previously described[38]. Long Evans males (residents), pre-screened for aggression and dominance, were housed in a large cage (61 cm X 43 cm X 20 cm) with females and progeny. During social defeat (21 days), the experimental male Wistar (intruder) was co-housed with the resident but physically separated by an acrylic partition that allowed for exchange of visual, auditory and olfactory information between the intruder and the residents. For 3 min each day, the female resident and pups were removed from the cage, and the partition was lifted to allow a direct physical interaction between the males. Social defeat was defined as a supine submissive posture of the intruder for 3 consecutive seconds with the resident

pinning the intruder down. After a social defeat encounter or 3 min (whichever occurred first), the intruder was removed and its reward threshold was measured. At the end of each day of testing, the intruder was paired with a different resident for the next 24 h period.

2.4.6 Drug treatment

Clozapine (0.1 mg/kg; MP Biomedicals, Santa Ana, CA) was dissolved in 0.1% dimethyl sulfoxide (DMSO) in sterile saline and administered intraperitoneally (IP) once daily, 30 min prior to ICSS testing during the 21-day social defeat procedure. For habituation to IP injections, rats across all experimental groups were administered 0.1 ml of 0.1% DMSO in saline (equal volume and route of administration as for subsequent clozapine injections) 30 min prior to ICSS testing daily, for 7-14 days prior to the start of social defeat until their ICSS baseline thresholds stabilized.

2.4.7 Tissue collection and processing

In all experiments, on day 21, 6 h after social defeat/ICSS testing, rats were administered 0.2-0.3 g/kg Fatal-Plus C IIN (pentobarbital sodium; IP). After complete loss of reflexes, rats were transcardially perfused with phosphate-buffered saline (PBS, pH 7.5) until perfusate was colorless, followed by perfusion with equal volume of ice-cold 4% paraformaldehyde (PFA, pH 7.5) dissolved in PBS. Rats were then decapitated and whole brains were extracted and post-fixed in fresh 4% PFA for 24 h at 4°C followed by incubation in 30% sucrose at 4°C for 48 h or until brains were completely submerged.

Thirty μm coronal sections of VTA, DR and 50 μm sections of the hypothalamus and amygdala were collected using a microtome (Leica) in a cryoprotectant solution (30% v/v glycerol, 30% v/v ethylene glycol in PBS, pH 7.4) for long-term storage at -20°C to -40°C. For each brain

region, every fourth section was collected in the same well as a set of tissue for a given staining procedure.

2.4.8 Immunohistochemistry and *in situ* hybridization

Antibody details and concentrations are provided in Table 2.1. All washes and incubations were performed with gentle shaking. Antibodies were diluted in blocking solution (5% normal horse serum, 0.3% Triton-X 100 in PBS). For immunofluorescence, sections were washed 3 times for 5' (3 x 5') in PBS, incubated in blocking solution for 30' to 1h, incubated in primary antibody solution overnight at 4°C, washed 3 x 10' in PBS, incubated in secondary antibody solution for 1h at room temperature, washed 3 x 10' in PBS, mounted on a positively charged glass slide (Fisherbrand Superfrost Plus) in 0.2% gelatin in PBS, coverslipped with mounting medium (Fluoromount-G®, SouthernBiotech) and sealed with nail polish.

For colorimetric DAB-based immunohistochemistry, brain sections were processed in the following steps: 3 x 5' PBS washes, incubation in 3% hydrogen peroxide in PBS for 15', blocking solution for 30', primary antibody overnight at 4°C, 3 x 10' PBS washes, secondary antibody incubation at room temperature, 3 x 10' PBS washes, incubation in fresh ABC solution (1:1 mixture of Reagents A and B, each diluted 1:100 in 2% NaCl/0.3% Triton-X 100/PBS) (VECTASTAIN® HRP Kit, Vector Labs), 3 x 10' PBS washes, incubation for 3-5' in 3,3' diaminobenzidine (DAB, Acros Organics) staining solution (0.025% w/v DAB, 0.01% v/v hydrogen peroxide in PBS), 3 x 10' PBS washes, mounting in 0.2% gelatin in PBS, drying and coverslipping in Cytoseal™ 60 (Thermo Scientific) and sealing with nail polish.

RNAscope® v 2.0 (ACD Bio) *in situ* hybridization in combination with immunofluorescence was performed as per manufacturer's instructions for *Tph2* (Part ID 316411), *Vglut3* (Part ID 476711-C2) and *Pet1* (*Fev*) (Part ID 487771-C3) mRNA transcripts.

Table 2.1: Antibodies

Primary and secondary antibodies used in this study listed with details of host and target species, type (polyclonal, monoclonal or secondary), working dilution and product supplier.

Antibody	Dilution	Catalog # (Manufacturer)
Mouse α cFos (monoclonal)	1:1000	sc-166940 (Santacruz Biotech)
Guinea pig α NeuN (polyclonal)	1:2000	ABN90 (Millipore Sigma)
Sheep α TH (polyclonal)	1:1000	AB1542 (Millipore Sigma)
Rabbit α TPH2 (polyclonal)	1:500	PA1-778 (Thermo Fisher Scientific)
Guinea pig α VGlut3 (polyclonal)	1:100	AB5421-I (Millipore Sigma)
Sheep α bNOS (polyclonal)	1:500	AB1529 (Millipore Sigma)
Donkey α sheep Alexa Fluor 647 (secondary)	1:500	A21448 (Invitrogen)
Donkey α sheep Alexa Fluor 488 (secondary)	1:500	A11015 (Invitrogen)
Donkey α guinea pig Alexa Fluor 488 (secondary)	1:500	706-545-148 (Jackson ImmunoResearch)
Donkey α rabbit Alexa Fluor 555 (secondary)	1:500	A31572 (Invitrogen), SAB4600177 (Sigma)
Horse α rabbit biotinylated (secondary)	1:300	BA-1100 Vector Laboratories, CA
Donkey α mouse Alexa Fluor 647 (secondary)	1:500	A31571 (Invitrogen)

2.4.9 Image acquisition and processing

For fluorescence staining, multi-channel confocal z-stacks of each tissue section were acquired with 2.5 μm distance between optical sections using a Leica SPE confocal microscope (10X or 20X dry objective). Objective resolution and acquisition settings (laser power, gain, pinhole aperture and signal averaging) were applied uniformly across sections within a given experiment. Maximum intensity z-projections were made using Fiji[170] image analysis software. Linear brightness and contrast adjustments were applied uniformly across all pixels for each image. Images in TIFF format were used for quantification. Mean filter (Fiji) or box blur (Adobe Photoshop CS4) with 2-pixel radius was applied to representative images in figures.

For DAB-stained sections, 20X brightfield images were acquired using an Olympus Virtual Slide Microscope (VS120) and stored in TIFF format for quantification.

2.4.10 Quantification of cell number

For quantification of cell numbers for each marker, fluorescence or brightfield images (processed as described above) were organized according to their rostrocaudal position and specific sections were chosen by visual inspection for quantification and analysis using the Rat Brain Atlas[171] as a reference. For quantification of TPH2, cFos, NeuN, VGLUT3, nNOS immunofluorescence and *Tph2*, *Vglut3* and *Pet1* mRNA in the mid-DR, sections at rostrocaudal positions -7.76, -7.88 and -8.00 mm from bregma were selected and subdivisions were demarcated based on TPH2 staining pattern with reference to Abrams et al.[172], Kelly et al.[173] and Paxinos and Watson[171].

For quantification of TPH2 using DAB staining, sections at rostrocaudal positions -7.64, -7.76, -7.88, -8.00, -8.12, -8.24 and -8.36 mm from bregma were chosen. Only cells that were in

focus, with clearly discernible cellular appearance (size, shape and cell boundaries) and with intense colorimetric or fluorescent stain filling at least 50% of the cell's area (by visual estimation), were considered positive.

For quantification of TH in the VTA, every fourth 30 μm section between rostrocaudal positions -5.60 mm and -6.20 mm from bregma was chosen. VTA was demarcated based on TH expression with reference to Paxinos and Watson[171]. Fluorescently stained cell bodies were counted unilaterally in the parabrachial pigmented and paranigral nuclei of the VTA and doubled prior to analysis and plotting.

For quantification of TH in the periventricular nucleus of the hypothalamus, every fourth 30 μm section between rostrocaudal positions -1.40 mm and -2.00 mm from bregma was chosen. The periventricular nucleus was identified by TH expression with reference to Paxinos and Watson[171] and cell bodies were counted unilaterally and doubled prior to analysis and plotting.

In all animals with chemogenetic manipulation, validation of DREADD receptor expression was performed by visually examining sections of the central amygdala (lateral subnucleus, bilateral) throughout its rostrocaudal extent, for native mCherry or eGFP expression under a fluorescence microscope.

2.4.11 Experimental design and statistical analyses

IBM SPSS Statistics 24 software was used for all statistical testing. Reward threshold comparisons for non-chemogenetic experiments were made using a mixed ANOVA (with Greenhouse-Geiser correction for sphericity violation as $\varepsilon<0.75$) after testing for normality (Shapiro-Wilk's test) and homogeneity of variance (Levene's test). *Day* was included as a within-

subjects factor and *Stress* (control, susceptible and resilient) was the between-subjects factor. Significant main and interaction effects were followed with Bonferroni *post hoc* tests.

For cell quantification comparisons in non-chemogenetics experiments, a 1-way ANOVA was used (control, susceptible and resilient groups) after ensuring normality and homoscedasticity of data (Shapiro-Wilk's test and Levene's test, respectively) and Tukey's HSD *post hoc* tests were performed if applicable. Data that did not satisfy criteria for an ANOVA were analyzed using Kruskal Wallis H tests and *post hoc* comparisons with Bonferroni correction were performed.

For the chemogenetics experiment, rats either expressed the GFP or hM3Dq virus and were split into control and defeat groups after their ICSS baseline thresholds stabilized. All rats were administered 0.1% DMSO in saline (vehicle) during habituation and clozapine during the 21d social defeat period. GFP- and hM3Dq virus expressing non-stressed controls were pooled after ensuring that there was no statistical difference between the groups. Effects of vehicle were tested using a 1-way repeated measures ANOVA (*Day* as within-subjects factor) that included all rats. Reward thresholds were compared using a mixed ANOVA with *Day* (for analysis of effects of vehicle and clozapine on control groups) or *Period* (acute: average of days 1-3 or chronic: average of days 19-21, for analysis of effect of clozapine on stressed groups) as the within-subjects factor and *Group* (Control, GFP susceptible, GFP resilient, hM3Dq susceptible and hM3Dq resilient) as the between-subjects factor. Significant main and interaction effects were followed with Bonferroni *post hoc* tests. Cell count comparisons between the same five groups were conducted using an ANOVA with Tukey's HSD for *post hoc* comparisons. The coefficient of correlation between TPH2+ counts and reward thresholds, was calculated using bivariate Pearson correlation test.

Alpha level was set to 0.05 for all analyses. Appropriate sample sizes for each experiment were determined with standard Cohen's d power analysis with target effect size set to 0.8 and alpha level to 0.05. Outliers within any group, determined using the median absolute deviation method[174] were excluded from statistical analyses. Microsoft Excel and GraphPad Prism 8.0.2 were used for generating plots.

2.5 Results

2.5.1 Susceptible rats show elevated ICSS thresholds after chronic social defeat

Monitoring ICSS thresholds over 21-days of social defeat revealed that a subset of rats was susceptible to stress-induced anhedonia, while others were resilient (Fig. 2.1A). Stressed rats whose thresholds at the end of the 21-day period, were greater than 3 standard deviations from baseline, were classified as 'susceptible' and others as 'resilient'. Independently, a k-means cluster analysis of thresholds at the end of social defeat (averaged over days 19-21), split the cohort of stressed rats into identical groups, with susceptible rats forming a separate cluster from resilient rats and unstressed controls (Fig. 2.1B, right). The same cluster separation was not present at the beginning of social defeat (averaged over days 1-3), indicating that susceptible/resilient phenotypes were not predictable early during social defeat, in response to acute exposure, but rather developed over time due to chronic stress (Fig. 2.1B, left). There were also no differences in baseline thresholds across groups (Fig. 2.1C) as determined by a Kruskal-Wallis H test ($\chi^2(2)=1.233$, $p=0.540$), indicating that baselines thresholds do not predict ICSS responses to subsequent social defeat. A 2-way mixed ANOVA using Greenhouse-Geiser correction ($\varepsilon=0.379$) revealed a main effect of *Stress* ($F(2,25)=15.998$, $p=3.40E-5$; Fig. 2.1A) but no significant interaction between *Stress* and *Day* ($p=0.106$). However, based on previous evidence that

susceptible/resilient phenotypes develop over time[38] which was supported by our observations (Fig. 2.1A,B), we hypothesized that the behavioral difference between susceptible and resilient rats arises at an intermediate timepoint during the 21-day social defeat paradigm. Accordingly, we performed *post hoc* pairwise comparisons with Bonferroni correction, which revealed that susceptible rats had significantly elevated thresholds ($p<0.05$) relative to controls on days 2-10 and relative to both resilient and control animals from day 11 onwards (Fig. 2.1A), while resilient rats did not differ significantly from controls.

While controls gained weight over time, all stressed rats showed a slight decrease in weight over the 21-day stress period, indicating that the metabolic effects of stress were similar across susceptible and resilient rats (Fig. 2.1D). Latencies to supine submissive posture during social defeat, a quantitative measure of stress exposure, were also similar between susceptible and resilient groups (Fig. 2.1E), indicating that resilient animals were not subjected to any less stress than susceptible rats. Rats across groups did not show a difference relative to controls in latency to respond to ICSS stimulation, indicating that social defeat did not differentially affect motor activity between susceptible, resilient, and control rats (Fig. 2.1F). Number of injuries during social defeat was also similar across susceptible and resilient rats (Fig. 2.1G), indicating that elevated thresholds in susceptible rats were likely not a function of immune responses to injury.

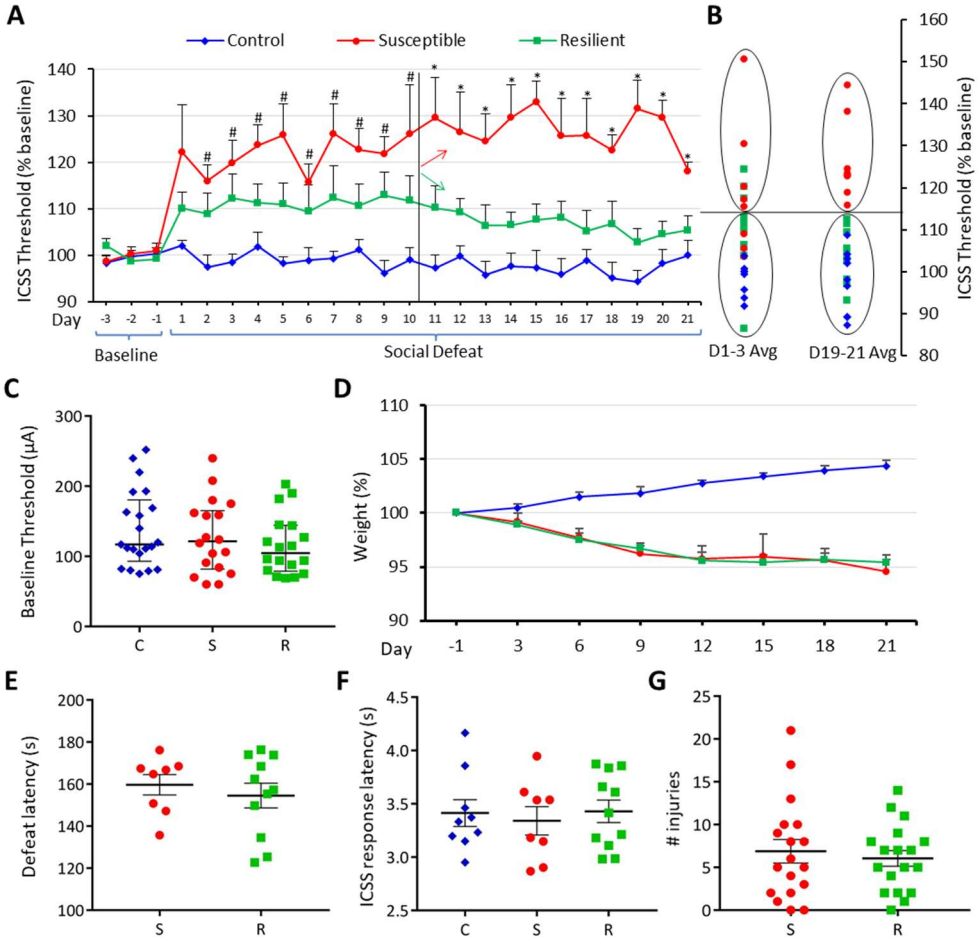


Figure 2.1: Susceptible rats show elevated ICSS thresholds during stress

A, Daily ICSS thresholds (mean across animals + s.e.m.) plotted as percent of baseline for 3 days prior to defeat and each day for 21-day social defeat. Control rats (*n=9*) represented by blue diamonds, susceptible rats (*n=8*) by red circles and resilient rats (*n=11*) by green squares. Significant ($p<0.05$) post-hoc pairwise comparisons for each day shown above error bars. # indicates significant difference between susceptible and control groups but not between other pairs. * indicates significant difference between susceptible and control as well as susceptible and resilient groups. Dashed vertical line after Day 10 indicates the day from which susceptible (red arrow) and resilient (green arrow) groups differ significantly. **B**, K-means cluster analysis of Days 1-3 (left) and Days 19-21 (right). **C**, Absolute baseline ICSS current intensity thresholds (in μA) for each experimental group (C, controls; S, susceptible; R, resilient). Values are median and interquartile range. **D**, Rat body weight (mean ± s.e.m.) in grams, measured every 3 days over 21-day period and plotted as percent change relative to baseline (Day -1) for each group. **E**, Latency to supine submissive posture during social defeat, in seconds, plotted for stressed groups as mean ± s.e.m. **F**, ICSS response latency (mean ± s.e.m.) in seconds, plotted for each group. **G**, Total number of injuries (mean ± s.e.m.) suffered by each rat during 21-day social defeat plotted for stressed group. For **B-E**, graph markers indicate experimental conditions as defined in **A**.

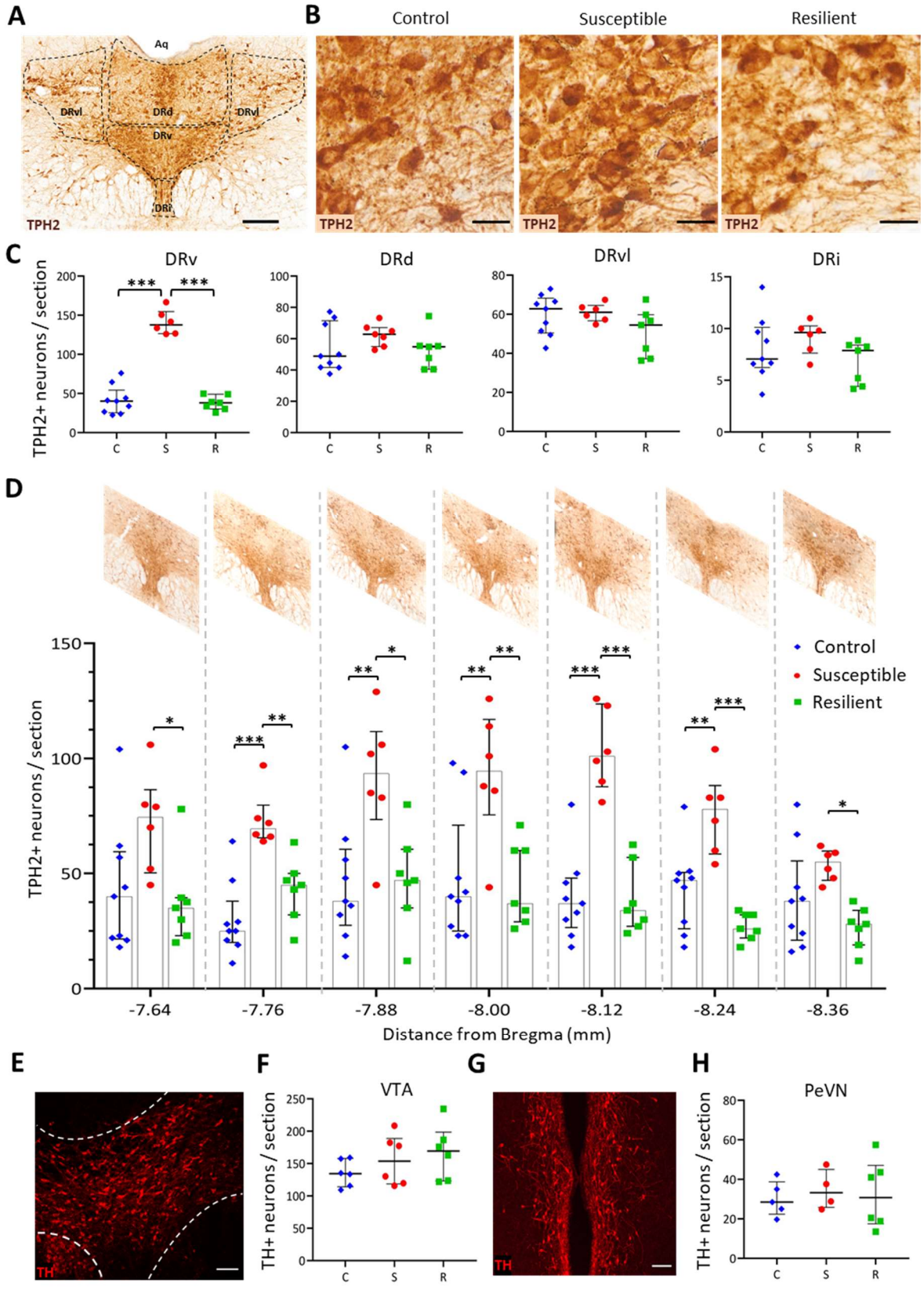
2.5.2 Susceptible rats display an increased number of TPH2+ neurons in the DRv

Altered numbers of TPH2+ neurons in the DR have been observed in human victims of suicide[102]. Therefore, we counted the number of TPH2+ neurons in each of the following subnuclei of the mid-rostrocaudal DR: dorsal (DRd), ventral (DRv), ventrolateral "wings" (DRvl) and interfascicular (DRi) (Fig. 2.2A). The number of TPH2+ neurons in the DRv was significantly elevated (Kruskal Wallis H test, $\chi^2(2)=11.528$, $p=0.003$) in susceptible rats, relative to control ($p=0.008$, Bonferroni's adjustment) and resilient rats ($p=0.008$, Bonferroni's adjustment) (Fig. 2.2B,C). The DRd, DRvl and DRi showed no differences in the average number of TPH2+ neurons per section across groups as determined by Kruskal Wallis H tests (Fig. 2.2C). Independent Kruskal Wallis H tests with *post hoc* pairwise comparisons using Bonferroni adjustment revealed a significantly increased number of TPH2+ neurons ($p<0.05$) in susceptible rats relative to resilient and control rats at each of the seven rostrocaudal positions examined (Fig. 2.2D), indicating that stress-induced TPH2 expression was not localized to particular rostrocaudal sub-regions of the DRv.

Since neurotransmitter plasticity involving dopaminergic neurons (marked by tyrosine hydroxylase, TH) has been observed in other brain regions, such as the periventricular nucleus (PeVN) of the hypothalamus after photoperiod stress[114] and the ventral tegmental area (VTA)[117] after neonatal exposure to nicotine, we investigated whether the number of TH+ neurons differed across our experimental groups in these regions. Neither of these regions showed differences across groups as determined by Kruskal Wallis H tests (Fig. 2.2E-H), indicating the specificity of TPH2 plasticity in the DR following social stress.

Figure 2.2: Susceptible rats display more TPH2+ neurons in the ventral subnucleus of the dorsal raphe nucleus (DRv)

A, Representative coronal section through the dorsal raphe nucleus (DR) stained for tryptophan hydroxylase isoform 2 (TPH2, brown) by DAB immunohistochemistry. Various DR subnuclei present at the mid-rostrocaudal level are outlined in black. DRd, dorsal; DRv, ventral; DRvl, ventrolateral; DRi, interfascicular; Aq, aqueduct of Sylvius. Scale bar: 200 μm. **B**, Representative images of DRv sections stained for TPH2 from each experimental group. Scale bars: 25 μm. **C**, Number of TPH2+ neurons in ventral (DRv), dorsal (DRd), ventrolateral (DRvl) and interfascicular (DRi) subnuclei of the DR. Counts (per section) were averaged across rats (Controls, C, blue diamonds, *n=9*; susceptible, S, red circles, *n=6*; resilient, R, green squares, *n=7*) and plotted as median with interquartile range. *** $p < 0.001$. **D**, Quantification of number of TPH2+ neurons in the DRv at 120 μm rostro-caudal intervals in the middle DR. Rostro-caudal positions represented on x-axis as distance from bregma in mm and representative images of the DR at each position are shown above. Counts were averaged across rats (graph symbols and sample sizes same as in **C**) and plotted as median and interquartile range. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **E**, Representative coronal section through the ventral tegmental area (VTA) seen unilaterally, outlined in white, stained for tyrosine hydroxylase (TH, red) to mark dopaminergic neurons. Scale bar: 100 μm. **F**, Bilateral quantification of the number of TH+ neurons per VTA section. Bilateral counts were averaged across 6 rats per group and plotted as median and interquartile range. Graph symbols as defined in **C**. **G**, Bilateral view of a representative coronal section through the periventricular nucleus of the hypothalamus (PeVN), stained for tyrosine hydroxylase (TH, red) to mark dopaminergic neurons. Scale bar: 100 μm. **H**, Bilateral quantification of number of TH+ neurons per PeVN section. Counts were averaged across rats (Controls *n=5*, susceptible *n=4*, resilient *n=6*) and plotted as median and interquartile range. Graph symbols as defined in **C**.


A
Aq
DRvl
DRd
DRvl
DRv
DRi
TPH2
B
Control
Susceptible
Resilient
TPH2
C
DRv
DRd
DRvl
DRi
TPH2+ neurons / section
C
S
R
D
Control
Susceptible
Resilient
TPH2+ neurons / section
-7.64
-7.76
-7.88
-8.00
-8.12
-8.24
-8.36
Distance from Bregma (mm)
E
TH
F
VTA
TH+ neurons / section
G
TH
H
PeVN
TH+ neurons / section

2.5.3 TPH2/VGLUT3 switching occurs in susceptible rats following chronic stress

We first asked if the increase in TPH2+ neurons came from an increased number of neurons in the DRv. No significant differences were observed in the total number of mature neurons (marked by NeuN) in the DRv, as measured by a 1-way ANOVA (Fig. 2.3A,B). To identify the reserve pool[108] of differentiated DRv neurons that is recruited to acquire TPH2 in susceptible rats, we examined DRv for expression of other neurotransmitters and asked whether the extent of their co-expression with TPH2 changed across experimental groups. Of the various neurotransmitters expressed in the DR[175], we chose to examine those previously implicated in stress and reward.

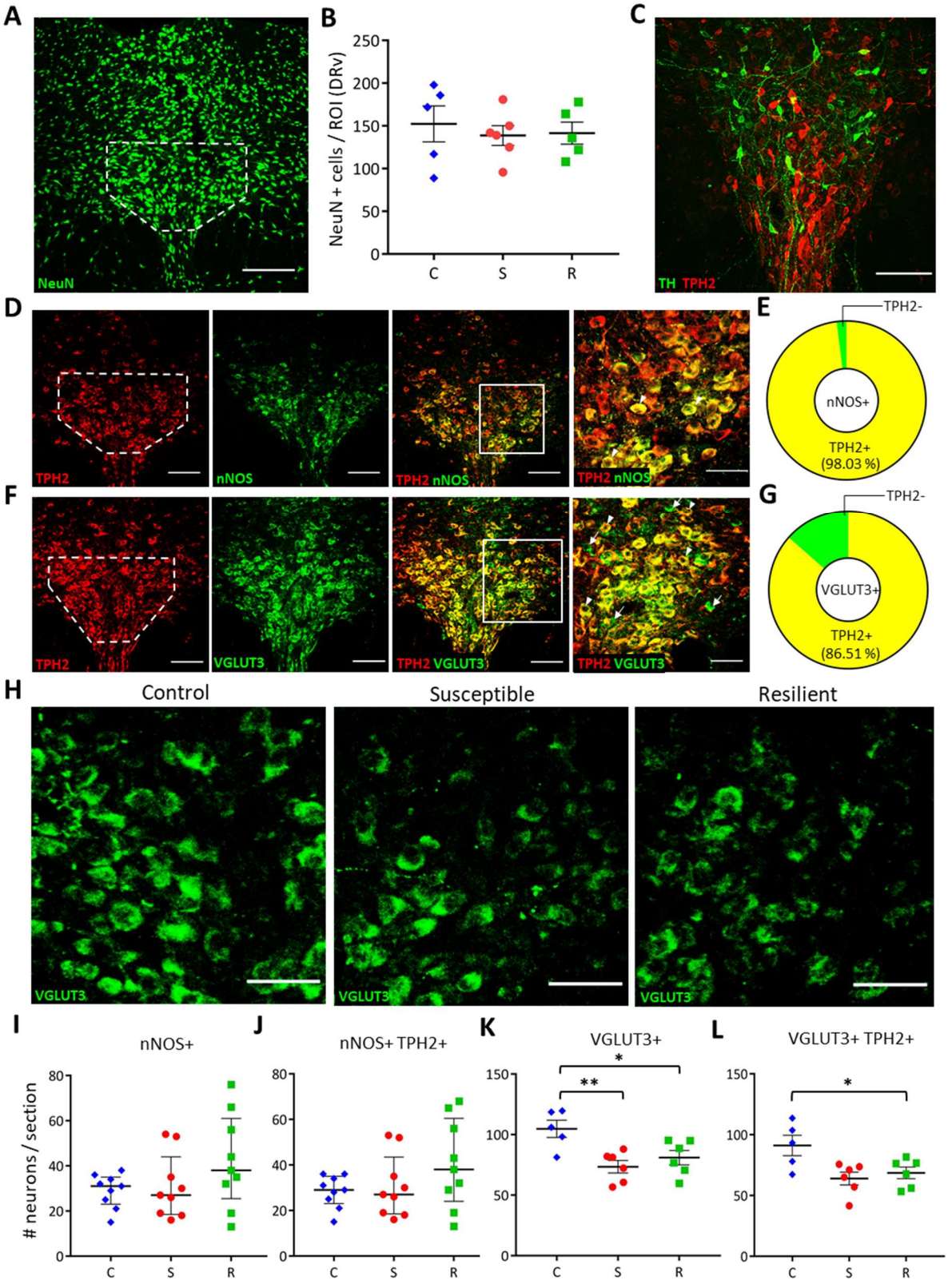
Mesolimbic dopamine is a well-known mediator of reward[176] and we investigated whether DR dopaminergic (TH+) neurons could display plasticity of TPH2 phenotype. TH+ neurons in the DR did not display any overlap with TPH2 expression and were located very rostrally within the DR, outside the region where increased TPH2+ neurons were observed (Fig. 2.3C), consistent with previous observations[175]. Another candidate neurotransmitter in the DR associated with reward and depression is nitric oxide[177–179]. Nitrergic neurons, marked by neuronal nitric oxide synthase (nNOS), were found to highly co-express TPH2 in the DRv (Fig. 2.3D,E) with 98.03 ± 1.44 % of nNOS+ neurons co-expressing TPH2 in non-stressed animals (Fig. 2.3E). There were no significant differences in the total number of nNOS+ neurons (Fig. 2.3I) or nNOS+ TPH2+ co-expressing neurons (Fig. 2.3J) across conditions. A 98 % TPH2+ nNOS+ co-expression indicated that there were not enough nNOS+ TPH2- neurons to account for the rise in TPH2+ numbers in susceptible rats.

VGLUT3-expressing neurons have been shown to co-express TPH2, project to the VTA, and drive reward[133]. Accordingly, we immunostained (Fig. 2.3F) and quantified the number of

DRv glutamatergic neurons, marked by vesicular glutamate transporter isoform 3 (VGLUT3) and found that 86.51 ± 2.78 % of VGLUT3+ neurons co-express TPH2 in control brains (Fig. 2.3G). We hypothesized that the remaining VGLUT3+ TPH2- neurons could represent a significant portion of the reserve pool available for stress-induced TPH2 recruitment. To test whether VGLUT3 is a 'switching partner' for TPH2 as previously shown for TH[113,114], we quantified the number of total VGLUT3+ and VGLUT3+ TPH2+ co-expressing neurons in the DRv. A 1-way ANOVA ($F(2,14)=7.001$, $p=0.008$) revealed a significant decrease in the number of VGLUT3+ neurons in both susceptible (Tukey's HSD: $p=0.039$) and resilient (Tukey's HSD: $p=0.007$) rats relative to controls (Fig. 2.3H,K). The number of VGLUT3+ TPH2+ co-expressing neurons was also decreased in stressed rats (Fig. 2.3L, 1-way ANOVA $F(2,14)=5.367$, $p=0.019$) with a significant difference between resilient and control groups (Tukey's HSD: $p=0.023$) and a strong trend toward a significant decrease in the susceptible group (Tukey's HSD: $p=0.054$).

Figure 2.3: Stressed rats have fewer VGLUT3+ neurons in the DRv

A, Representative coronal DR section showing NeuN immunoreactivity. DRv margins were outlined in white. Scale bar: 200 μm. **B**, Quantification of NeuN+ cells in control (C, blue diamonds, *n=5 rats*), susceptible (S, red circles, *n=6 rats*), and resilient (R, green squares, *n=5 rats*) groups. ROI, region of interest. Counts were normalized to ROI area, averaged across rats per group and plotted as mean ± s.e.m. **C**, Representative coronal DR section showing dopaminergic (TH+, green) and serotonergic (TPH2+, red) but no co-expression. Scale bar: 100 μm. **D**, Representative images of a coronal DR section showing nitrergic (nNOS) neurons co-expressing TPH2. DRv outlined in white. Left to right: TPH2 (red), nNOS (green), merge (scale bar: 100 μm), and higher magnification of ROI drawn in merged image (scale bar: 25 μm). Arrowheads indicate nNOS+ TPH2+ (yellow) co-expressing neurons. **E**, Quantification of TPH2/nNOS co-expression in the DRv. Yellow sector indicates percentage of nNOS+ neurons co-expressing TPH2. Green sector indicates nNOS-only neurons. **F**, Representative images of coronal DR section showing glutamatergic (VGLUT3+) neurons co-expressing TPH2. DRv outlined in white. Left to right: TPH2 (red), VGLUT3+ (green), merge (scale bar: 100 μm), and higher magnification of ROI drawn in merged image (scale bar: 50 μm). Arrowheads indicate VGLUT3+ TPH2+ (yellow) co-expressing neurons. **G**, Quantification of TPH2/VGLUT3 co-expression in the DRv. Yellow sector indicates percentage of VGLUT3+ neurons also expressing TPH2. Green sector indicates VGLUT3-only neurons. **H**, VGLUT3+ neurons in the DRv of control, susceptible and resilient groups (scale bar 50 μm). **I**, **J,** Quantification of nNOS+ (**I**) and nNOS+ TPH2+ co-expressing (**J**) neurons in the DRv of control (C, blue diamonds), susceptible (S, red circles) and resilient (R, green squares). Counts were obtained from 3 sections each from 3 rats per group and plotted as median with interquartile range. **K**, Quantification of DRv VGLUT3+ neurons. **L**, Quantification of DRv VGLUT3+ TPH2+ co-expressing neurons. For **K** and **L**, counts were averaged across rats (Controls *n=5*, susceptible *n=6*, resilient *n=6*) and plotted as mean ± s.e.m. * $p < 0.05$, ** $p < 0.01$. Graph symbols as defined in **I**.


A
NeuN
B
NeuN + cells / ROI (DRv)
250
200
150
100
50
0
C
S
R
C
TH TPH2
D
TPH2
nNOS
TPH2 nNOS
TPH2 nNOS
E
TPH2-
nNOS+
TPH2+
(98.03 %)
F
TPH2
VGLUT3
TPH2 VGLUT3
TPH2 VGLUT3
G
TPH2-
VGLUT3+
TPH2+
(86.51 %)
H
Control
Susceptible
Resilient
VGLUT3
VGLUT3
VGLUT3
I
nNOS+
neurons / section
80
60
40
20
0
C
S
R
J
nNOS+ TPH2+
K
VGLUT3+
*
**
150
100
50
L
VGLUT3+ TPH2+
*

To test whether the observed changes in TPH2 and VGLUT3 protein arose from changes in the corresponding mRNA, we performed *in-situ* hybridization for *Tph2* and *Vglut3* mRNA (Fig. 2.4A). Additionally, we also probed for *Pet1* mRNA (Fig. 2.4A), which encodes a key transcription factor controlling serotonergic identity[180]. PET1 regulates transcription of *Tph2*, *Sert* and other components of serotonergic identity[89]. As expected, we found that all neurons expressing *Tph2* mRNA also expressed *Pet1* mRNA (Fig. 2.4C,D; arrowheads); however, Kruskal Wallis H tests revealed no significant differences across experimental groups in levels of *Tph2*, *Vglut3* or *Pet1* mRNA (Fig. 2.4B). Interestingly, a small fraction (9.17 ± 2.99 %) of *Pet1*-expressing neurons did not contain *Tph2* mRNA; a further subset (66.66 ± 11.33 %) of which co-expressed *Vglut3* mRNA (Fig. 2.4D; dashed outlines). The *Pet1*+ *Tph2*- neurons (both glutamatergic and non-glutamatergic) might represent an additional reserve pool, that is not recruited by 21-day social defeat, but might be primed for acquisition of *Tph2* mRNA and TPH2 protein when induced by prolonged chronic stress, extending beyond 3 weeks.

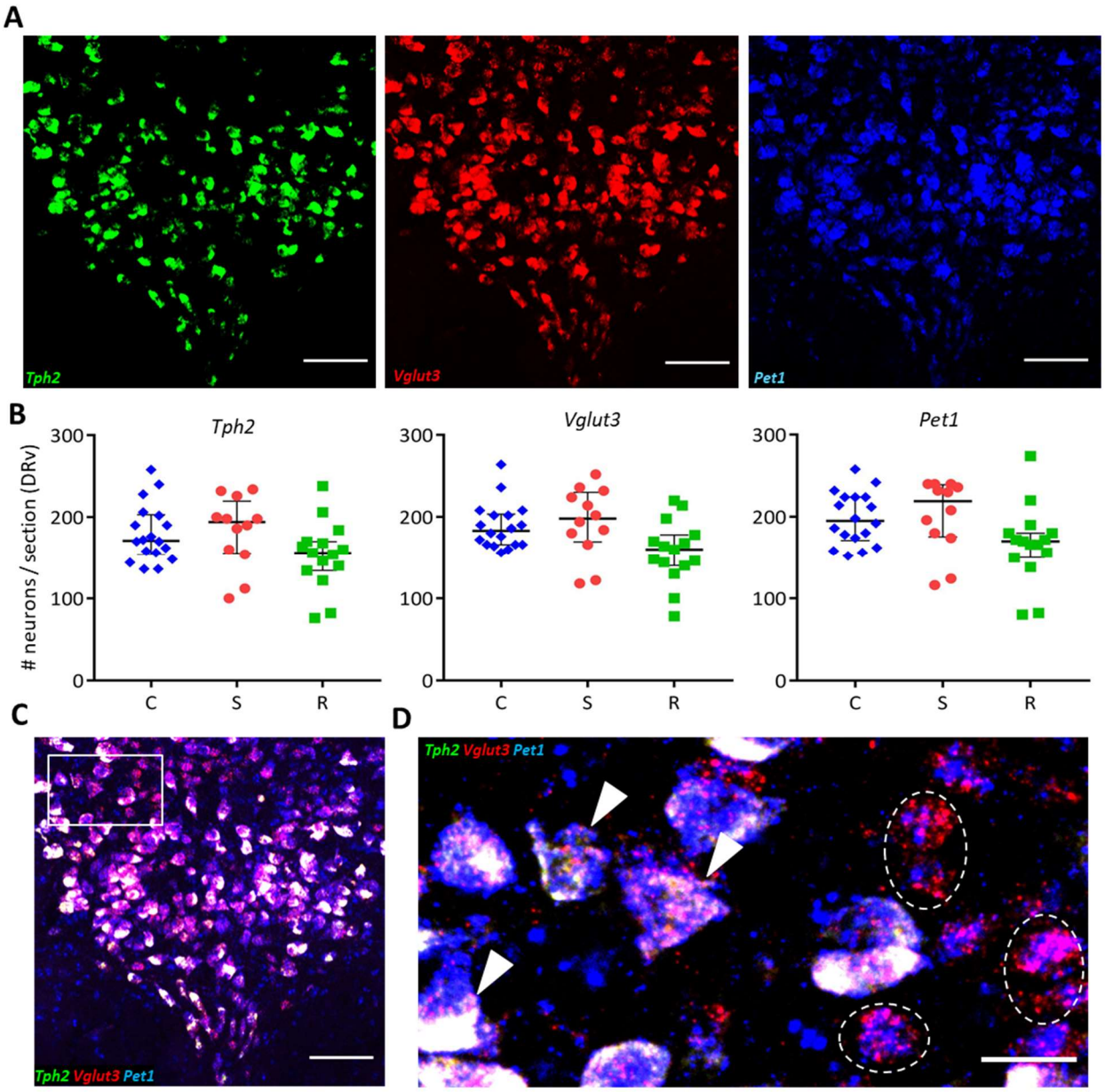


Figure 2.4: Levels of *Tph2*, *Vglut3* and *Pet1* mRNA do not differ across stress conditions.

A, Representative images of a coronal DR section processed for *Tph2* (green), *Vglut3* (red) and *Pet1* (blue) triple *in* situ hybridization. Scale bars: 100 µm. **B**, Quantification of the number of neurons expressing *Tph2*, *Vglut3* and *Pet1* mRNA across control (C, blue diamonds, *n=5 rats*), susceptible (S, red circles, *n=4 rats*) and resilient (R, green squares, *n=4 rats*) groups. Counts were obtained from 1-3 DRv sections from each rat and plotted as median with interquartile range. **C**, Merge of 3 channels shown in **A**. Scale bar: 100 µm. **D**, ROI (rectangle in **C**) at higher magnification showing triple labelled (arrowheads), and non-serotonergic *Pet1+ Vglut3+ Tph2-* (dashed outline) neurons. Scale bar: 25µm.

2.5.4 Stressed rats display lower cFos expression in DRv neurons

Neurotransmitter plasticity is activity-dependent[110,112,181,182]. To investigate whether the recruitable reserve pool of TPH2- neurons in the DRv experiences any change in neuronal activity in response to social defeat, we assessed cFos expression by immunohistochemistry across stress conditions (Fig. 2.5A). The total number of cFos-immunoreactive neurons was decreased following stress (Fig. 2.5A,C; Kruskal Wallis H test, $\chi^2(2)=11.325$, $p=0.003$). *Post hoc* pairwise comparisons with Bonferroni correction showed that the decrease in cFos was significant in susceptible ($p=0.004$) and resilient ($p=0.044$) rats relative to controls. Specifically, this decrease occurred in TPH2- neurons (Fig. 2.5B,D; Kruskal Wallis H test, $\chi^2(2)=17.967$, $p<0.001$), both in susceptible ($p<0.001$) and resilient ($p=0.031$) rats, while cFos expression was unchanged in TPH2+ neurons (Fig. 2.5E). The observed differences in cFos expression across groups indicated that neuronal activity changed specifically in TPH2- DRv neurons in response to social stress. We next tested whether activity manipulation in these neurons results in alteration of their TPH2 phenotype, ultimately modulating behavior.

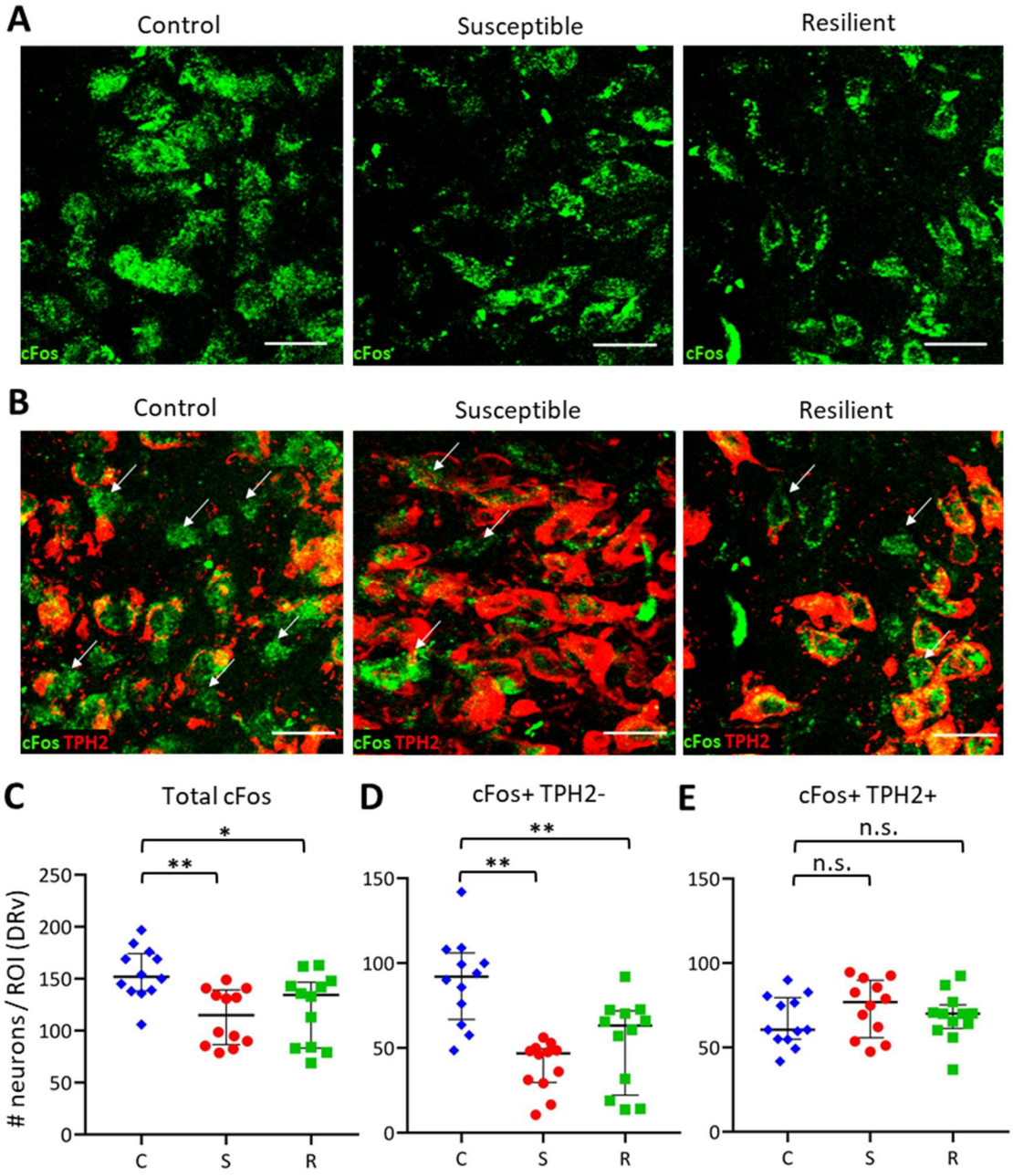


Figure 2.5: Stressed rats have fewer active TPH2- neurons in the DRv

A, cFos immunoreactivity in DRv of control, susceptible and resilient rats. Scale bars: 25 µm. **B**, cFos immunoreactivity in TPH2+ (red) and TPH2- (arrows) neurons in DRv across groups. Scale bars: 25 µm. **C**, **D**, **E**, Quantification of total cFos+ (**C**), cFos+ TPH2- (**D**), and cFos+ TPH2+ (**E**) neurons in controls (C, blue diamonds), susceptible (S, red circles), and resilient (R, green squares). Counts were obtained from 4 sections each from 3 rats per group, normalized to ROI area, and plotted as median and interquartile range. *$p < 0.05$, **$p < 0.01$, n.s. not significant (p>0.05).

2.5.5 Chronic activation of amygdalar CRH+ neurons during stress ameliorates anhedonia and prevents TPH2 induction

To investigate whether TPH2 plasticity is activity-dependent and whether resilience is inducible in animals subjected to social stress, we manipulated neuronal activity in the DRv, by using DREADDs to drive the activity of one of its major inputs[183,184], the central amygdala (CeA) (Fig. 2.6A). In *Crh-Cre* transgenic rats[169], the CeA was bilaterally transfected with a Cre-dependent AAV vector encoding the excitatory DREADD receptor (hM3Dq) tagged with mCherry (Fig. 2.6A,B). A Cre-dependent AAV vector expressing GFP was used as a control (Fig. 2.6C). Rats were then trained and baselined in the ICSS procedure, habituated to intraperitoneal injections of saline and tested for anhedonia during 21-day social defeat with daily clozapine pretreatment.

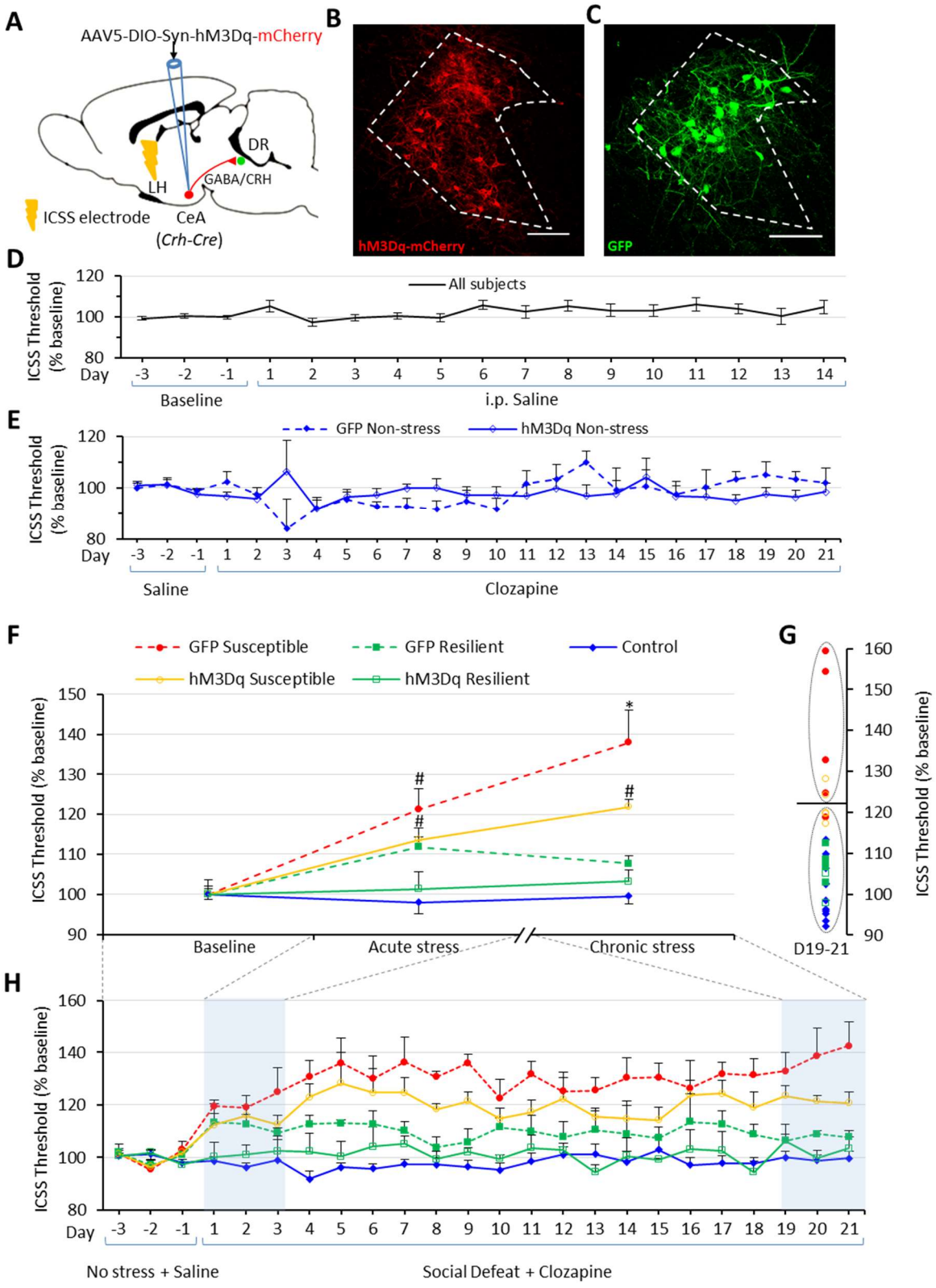
One-way repeated measures ANOVA with Greenhouse-Geiser correction revealed that saline injections did not significantly alter reward thresholds (Fig. 2.6D). To test whether clozapine by itself affected reward thresholds relative to baseline over the 21-day period, the non-stressed control group expressing GFP virus was examined using a 1-way repeated measures ANOVA with Greenhouse-Geiser correction. The within-subjects effect of *Day* was not significant (Fig. 2.6E). To test whether DREADD-mediated activation of the CeA affected reward thresholds in the absence of stress, thresholds of non-stressed controls expressing DREADDs and treated with clozapine for the 21-day period, were analyzed similarly. No significant effect of *Day* was observed (Fig. 2.6E), indicating that any effect of DREADD activation on reward thresholds in stressed rats was specific to the stress response and not a general reward-enhancing or reward-diminishing effect of stimulating CRH neurons of the CeA. A 2-way mixed ANOVA with Greenhouse-Geiser correction revealed that the two groups of non-stressed controls (GFP or hM3Dq virus injected) did not differ significantly from each other. Therefore, the two control

groups were combined and used as a pooled control group for all subsequent analyses. Based on previous evidence that susceptibility or resilience manifests after *chronic,* but not *acute*, stress[38] which was supported by our observations (Fig. 2.1A,B), we analyzed the acute (days 1-3) and chronic (days 19-21) effects of social defeat on reward thresholds using a 2-way mixed ANOVA (no violation of sphericity assumption, ε=1), with *Period* (acute or chronic) as the within-subjects factor and *Group* as the between-subjects factor (Fig. 2.6F). There was a significant interaction of *Period* x *Group* ($F(4,24)=2.994$, $p = 0.039$) and significant main effects of *Period* ($F(1,24)=5.329$, $p = 0.030$) and *Group* ($F(4,24)=16.922$, $p = 1.0E-6$) (Fig. 2.6F).

Pairwise *post hoc* comparisons with Bonferroni correction showed that GFP susceptible and hM3Dq susceptible rats had significantly elevated reward thresholds relative to non-stressed controls after acute stress exposure (GFP susceptible: $p=0.001$, hM3Dq susceptible: $p=0.032$). However, as hypothesized, the effects of chronic stress differed from those of acute stress across groups. After chronic stress (days 19-21), while the reward thresholds of GFP susceptible rats were significantly elevated relative to controls ($p=5.51E-7$) and GFP resilient rats ($p=0.001$), the reward thresholds of hM3Dq susceptible rats were not significantly elevated relative to the hM3Dq resilient group ($p=0.115$), but only elevated relative to control ($p=0.001$). In other words, the reward thresholds of the DREADD-treated susceptible rats were statistically similar to the resilient group, suggesting that DREADD-activation of CeA CRH+ neurons ameliorated chronic stress-induced anhedonia. A k-means cluster analysis (based on averaged thresholds of days 19-21) classified all controls and resilient rats into a single cluster (Fig. 2.6G). This cluster also included 3 out of 5 hM3Dq susceptible, and 1 out of 5 GFP susceptible rats. Other rats from the GFP susceptible and hM3Dq susceptible groups formed a separate cluster. Extended (day-wise) reward thresholds during chemogenetic manipulation are shown in Figure 2.6H.

Figure 2.6: Chronic activation of amygdalar CRH+ neurons reduces stress-induced reward threshold elevations

A, Cartoon showing viral strategy to chemogenetically activate CRH/GABA neurons of the central amygdala (CeA) in *Crh-Cre* rats. LH, lateral hypothalamus; DR, dorsal raphe nucleus. **B**, Expression of Cre-dependent mCherry-tagged excitatory DREADD (hM3Dq) virus in a *Crh-Cre* rat. Cell bodies and fibers localized to the lateral subnucleus of the central amygdala (CeL, dashed outline) are visible in red. **C**, Expression of Cre-dependent GFP virus in CeL (dashed outline) of a *Crh-Cre* rat. Scale bars in **B**,**C**: 100 μm. **D**, Daily ICSS thresholds (mean across rats ± s.e.m.) plotted as percent of baseline for 3 days prior to saline i.p. injections and each day for saline treatment. All subjects (*n=29 rats*) are plotted. **E**, Daily ICSS thresholds (mean across rats + s.e.m.) plotted as percent of baseline, for 3 days of baseline prior to clozapine treatment and each day of 21-day clozapine treatment in non-stressed controls. GFP-expressing rats (solid diamonds, dotted line, *n=4*) and hM3Dq-expressing rats (hollow diamonds, solid line, *n*=8) are plotted. **F**, ICSS thresholds plotted as percent of baseline (mean across rats +/- s.e.m.) for baseline (average of days -3 to -1) with saline treatment, acute (average of days 1-3) and chronic (average of days 19-21) social defeat with clozapine treatment. GFP-expressing susceptible rats (red solid circles, dotted line, *n=5*), GFP-expressing resilient rats (green solid squares, dotted line, *n=4*), hM3Dq-expressing susceptible rats (orange hollow circles, solid line, *n=5*) and hM3Dq-expressing resilient rats (green hollow squares, solid line, *n=3*) are plotted. Significant ($p<0.05$) post-hoc pairwise comparisons for each timepoint are indicated above error bars. # indicates significant difference relative to control but not to other groups. * indicates significant difference relative to control and resilient groups expressing the same virus. **G**, k-means cluster analysis of Days 19-21 averaged ICSS thresholds. Sample sizes and graph symbols as in **F**. **H**, Effect of DREADD stimulation of CeA CRH+ neurons on ICSS thresholds during social defeat. Daily ICSS thresholds (mean across rats + s.e.m.) plotted as percent of baseline, for 3 days of baseline prior to clozapine treatment/social defeat and each day of 21-day clozapine treatment/social defeat. Graph symbols as in **F**. Days used to compute averages for acute and chronic stress in **F** are highlighted in blue.


A
AAV5-DIO-Syn-hM3Dq-mCherry
DR
LH
GABA/CRH
ICSS electrode
CeA
(Crh-Cre)
B
hM3Dq-mCherry
C
GFP
D
ICSS Threshold (% baseline)
All subjects
Day
Baseline
i.p. Saline
E
GFP Non-stress
hM3Dq Non-stress
Saline
Clozapine
F
GFP Susceptible
GFP Resilient
Control
hM3Dq Susceptible
hM3Dq Resilient
Baseline
Acute stress
Chronic stress
G
D19-21
H
No stress + Saline
Social Defeat + Clozapine

At the anatomical level, the number of DRv TPH2+ neurons showed significant differences across groups (Fig. 2.7A; $F(4,24)=6.337$, $p=0.001$). *Post hoc* pairwise comparisons showed that the GFP susceptible group had a significantly elevated number of TPH2+ neurons relative to controls (Tukey's HSD: $p=0.041$), hM3Dq resilient (Tukey's HSD: $p=0.014$) and GFP resilient (Tukey's HSD: $p=0.001$) groups, recapitulating the effects of social defeat in wildtype rats (Fig. 2.2D) and demonstrating that clozapine alone had no effect on neurotransmitter plasticity in the DR following social defeat. Interestingly, the hM3Dq susceptible group did not display an elevated number of TPH2+ neurons relative to control (Tukey's HSD: $p=9.99E-1$), hM3Dq resilient (Tukey's HSD: $p=0.620$), or GFP resilient (Tukey's HSD: $p=0.142$) groups, indicating that manipulation of DR activity via CeA activation prevented stress-induced increase in TPH2+ neuron number. The lower extent of anhedonia in the hM3Dq susceptible rats, compared to GFP susceptible rats (Fig. 2.6F,G) is reflected in the lower TPH2+ neuron numbers in the hM3Dq susceptible group (Fig. 2.7A). TPH2+ neuron number in the DRv and ICSS thresholds were significantly positively correlated in all stressed rats (wildtype and transgenic) used in this study (Fig. 2.7B; Pearson's $r=0.743$, $p=3.0E-6$, $n=30$). This suggests that the number of TPH2+ neurons in the DRv is a molecular marker of susceptibility to anhedonia induced by chronic stress in rats.

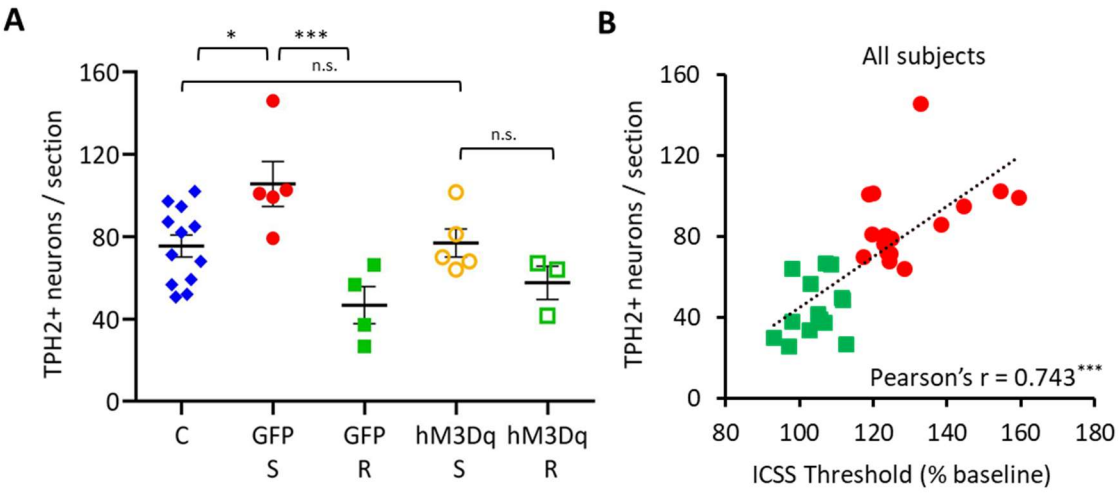


Figure 2.7: Chronic activation of amygdalar CRH+ neurons abolishes stress-induced gain of TPH2 in susceptible rats.

A, Quantification of TPH2+ neurons in the DRv from pooled control (C, blue diamonds, *n=12 rats*), GFP-expressing susceptible (GFP-S, red solid circles, *n=5 rats*), GFP-expressing resilient (GFP-R, green solid squares, *n=4 rats*), hM3Dq-expressing susceptible (hM3Dq-S, orange hollow circles, *n=5 rats*) and hM3Dq-expressing resilient (hM3Dq-R, green hollow squares, *n=3 rats*) groups. Counts were averaged across rats and plotted as mean ± s.e.m. * $p < 0.05$, *** $p < 0.001$, n.s. not significant ($p > 0.05$). **B**, Correlation analysis of DRv TPH2+ neurons and ICSS thresholds (Average of Days 19-21) for all stressed rats (wildtype and *Crh-Cre*) used in this study. Rats were classified as susceptible (red solid circles, *n=16*) or resilient (green solid squares, *n=14*), across genotypes and virus groups. Pearson's product-moment correlation coefficient shown on bottom right for each analysis. *** $p < 0.001$.

2.6 Discussion

Previous studies in rodent models have shown that serotonergic molecular machinery is upregulated in the DR after both chronic[151,152,185] and acute stress[150]. However, these studies did not specifically address differences between animals that are susceptible or resilient to chronic stress-induced anhedonia, or whether this upregulation occurs in identified classes of neurons expressing specific neurotransmitters. Other molecular adaptations investigated in the context of susceptibility/resilience such as synaptic excitability in the nucleus accumbens[51,71], VTA[51], medial

prefrontal cortex[186,187] and HPA axis activation[188] have not been demonstrated to be specific to anhedonia. Furthermore, neurotransmitter plasticity[108] as a mechanism to explain susceptibility/resilience to anhedonia has not been previously explored.

Our results revealed that chronic social defeat induced an increase in the number of TPH2+ neurons without affecting the total number of DRv neurons in susceptible rats, indicating that differentiated TPH2- neurons in the DR are recruited to additionally express TPH2. A concomitant reduction in VGLUT3+ neurons in the DRv of all stressed rats suggests that stress-induced anhedonia in susceptible animals is an example of transmitter switching driven by stress. Our finding is the first instance of a molecular and cellular marker based on neurotransmitter phenotype to be associated with vulnerability to stress. Neurotransmitter plasticity, also referred to as neurotransmitter switching[108], has been shown to occur in response to other forms of stress such as altered photoperiod exposure[114,115] or exposure to psychostimulants such as nicotine [117] and methamphetamine[116] . Interestingly, the change in number of TPH2+ neurons occurred exclusively in the DRv, but not in the other subnuclei of the mid-rostrocaudal DR. This is consistent with what is known about the afferent and efferent connectivity of the DRv. The DRv receives inputs from the lateral hypothalamus, central amygdala and orbito-frontal cortex[189] and projects to orbito-frontal and other cortical areas[190] and the VTA[191]. All of these areas are implicated either in reward sensing and integration[192–194], reward-based decision making[195], or stress processing[196–198]. The DRv is therefore, a critical hub that regulates stress and reward-related behaviors by serotonergic neuromodulation of multiple target areas. Neurotransmitter plasticity in the DRv could impact the activity and function of these regions, subsequently affecting behavior.

The increase in number of TPH2+ neurons in the anhedonic condition (susceptible animals), was somewhat unexpected, given that serotonergic depletion is conventionally

associated with depression based on studies that used SSRI treatment[161,199], tryptophan treatment[200–202] or 5-HT depletion[203–205]. However, increased TPH2 immunoreactivity has been observed after exposure to stress[206] and in brains of depressed patients who committed suicide[102,166], suggesting that this may be a homeostatic mechanism by the DR to compensate for serotonin depletion and deficient signaling in target areas. There is also ample electrophysiological evidence to suggest that serotonin exerts complex neuromodulation of the VTA in conjunction with glutamate and dopamine[131,133,207–210]; therefore an increase in TPH2+ neurons in the DRv may lead to reduced reward function in the VTA.

The opposing regulation of TPH2 and VGLUT3 observed in susceptible animals and the parallel finding that some VGLUT3+ neurons already express *Pet1* transcripts in unstressed animals, suggest that this glutamatergic pool might be primed for TPH2 recruitment, similar to the mechanism described for nicotine-induced dopaminergic plasticity within a Nurr1-expressing reserve pool in the VTA[117]. Since the number of neurons gaining TPH2 in susceptible animals is greater than the number of neurons losing VGLUT3, the existence of additional non-serotonergic neurons comprising the total reserve pool is expected. A fraction of GABA-expressing neurons in DRv[175,211] could be one such additional reserve pool. The absence of changes in *Tph2 or Vglut3* at the transcript level (Fig. 2.4) suggests that the observed differences in the number of TPH2+ and VGLUT3+ neurons across groups (Fig. 2.3) arose from differences at the level of post-transcriptional or translational regulation similar to miRNA-mediated dopamine/GABA switching in response to social cues in the developing amphibian olfactory bulb[212].

Neuronal activity, as measured by cFos expression, was decreased specifically in DRv TPH2- neurons following chronic stress, suggesting DRv inhibition after chronic stress. This is consistent with previous observations of serotonergic induction following inhibition of neuronal

activity[167]. The DRv receives projections from the central amygdala (CeA) containing GABA and corticotropin releasing hormone (CRH), which modulate the activity of both serotonergic and non-serotonergic neurons[169,183,184] in the DR, via CRHR1 and CRHR2 receptors[185,213,214]. Moreover, the CeA responds differently to acute versus chronic social defeat[215,216]. Further, CRH in particular is implicated in the development of depression-like phenotypes in rodents in response to uncontrollable stressors[217]. These reports which clearly indicated that the CRH+ neurons in the CeA form a strong input to the DRv involved in regulation of stress and reward-related behavior, motivated our choice to manipulate their activity. Lack of a significant difference between hM3Dq-treated susceptible and resilient rats at the end of chronic social defeat (days 19-21), suggested an antidepressant effect of activating CeA CRH+ neurons. It is likely that other brain regions involved in stress-processing and resilience, such as the medial prefrontal cortex[218], play a role in inducing resilience. Simultaneous activation of such regions might result in stronger effects on reward thresholds and resilience than what was achieved with CeA manipulation alone. The lack of a significant increase in the number of DRv TPH2+ neurons observed in hM3Dq-treated rats exposed to social defeat revealed the activity-dependent nature of resilience and its association with neurotransmitter phenotype in the DRv.

In conclusion, our findings begin to reveal a possible model of neurotransmitter plasticity involved in the regulation of resilience to social stress (Fig. 2.8). Susceptible animals gain TPH2 expression in the DRv in response to chronic stress, while resilient animals do not. VGLUT3 is lost in susceptible and resilient animals, largely by neurons that co-express both markers. This plasticity of expression occurs at the protein level, presumably by post-transcriptional or translational regulation since the number of neurons making the corresponding mRNA transcripts was unchanged. Activation of CRH+ neurons in the CeA, which form a major input to the DR,

modulates the effects of social stress on DR activity by preventing TPH2+ induction. This consequently prevents or ameliorates anhedonia, promoting resilience.

Knowledge of the molecular signature of the reserve pool neurons that are recruited to a TPH2 phenotype in animals susceptible to chronic-stress and the activity-dependent induction of resilience could both be harnessed in the future to develop novel treatment strategies to elicit resilience and ameliorate stress-related disorders.

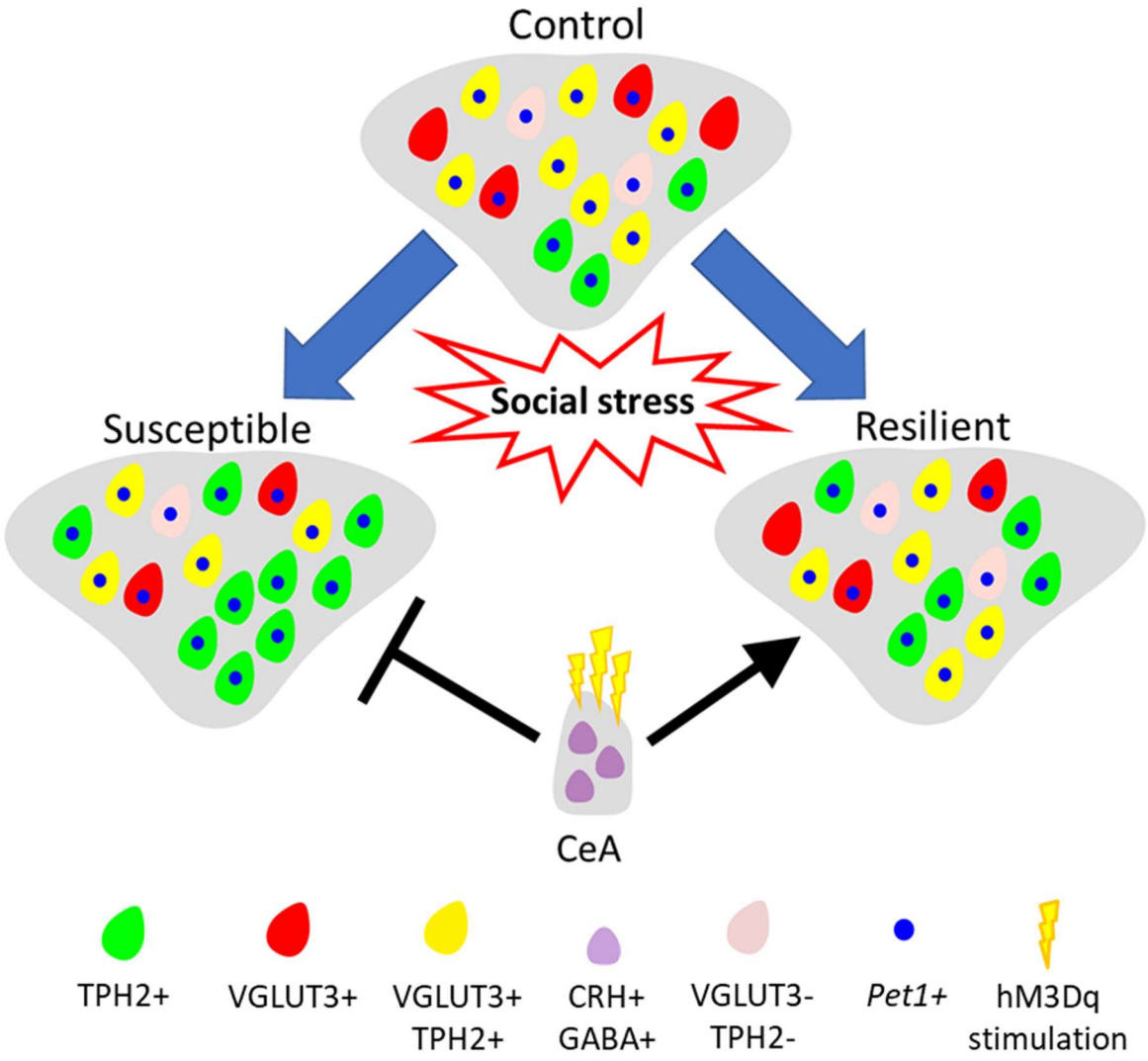


Figure 2.8: Model of transmitter plasticity in the DRv in response to chronic social stress

In response to chronic social stress, neurotransmitter plasticity occurs in the DRv of stressed rats. Rats susceptible to stress-induced anhedonia gain TPH2 and lose VGLUT3 while resilient rats only lose VGLUT3. Loss of VGLUT3 leads to lower co-expression of the two markers in both conditions. The plasticity occurs in neurons expressing *Pet1* transcripts. Activation of CRH+ neurons of the CeA promotes resilience and blocks susceptibility.

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CHAPTER 3

Future Directions

The findings described in Chapter 2 are evidence for the occurrence of neurotransmitter plasticity as a neurobiological phenomenon characterizing susceptibility and resilience to stress-induced anhedonia. As mentioned in Chapter 1, studies over four decades have documented various neural mechanisms underlying the processing of stress and reward, the role of serotonin and antidepressants, and the regulation of mood and emotion; yet the etiology and pathogenesis of depressive disorders remain to be fully understood. The discovery of neurotransmitter plasticity, as another weapon in the arsenal of homeostatic mechanisms regulating susceptibility to stress-induced pathologies, is an additional intriguing dimension to our understanding of the nervous system, its function and dysfunction.

This chapter discusses some further questions raised by the findings described in Chapter 2 that could motivate future research. Experimental approaches to answer these questions are also mentioned. Prior to discussing future directions, a few methodological considerations and potential improvements are noted in the following section.

3.1 Methodological considerations and potential improvements

3.1.1 Model organism

Rats (*Rattus norvegicus*) are a species favored by neurobiologists for their robust, reproducible repertoire of behaviors that parallel human behavior, the availability of past histological and electrophysiological data in the model, the ease of their rearing and maintenance and the relative ease of surgical manipulation of their brains for lesioning, implantation of

cannulae, microdialysis probes, optic fibers, and stimulating and recording electrodes. In fact, these reasons motivated the use of rats in this study to model stress-induced anhedonia.

However, for genetic studies and manipulation, the mouse (*Mus musculus*) is better suited as it has been used historically for forward and reverse genetics, resulting in the availability of a large number of transgenic mouse strains, high-resolution genetic and epigenetic information and tools for manipulation. Apart from molecular genetics, critical advances in electrophysiology, *in vivo* imaging and behavior are also mostly conducted in mice. Histological and biochemical techniques can be applied with equal ease to mice and rats. Mice can be housed at greater density compared to rats, which speeds up data collection in large cohorts.

The experimental design required to pursue some of the research questions discussed in subsequent sections need mice as the model because the requisite transgenic lines are not available in rats. Since several studies of stress, depression, susceptibility/resilience and neurotransmitter plasticity have already been conducted in mice, there are enough resources available to port the experiments described in this study to a mouse model, replicate the findings there and pursue further investigations in mice. By acquiring customized testing equipment, aggressor mouse strains and mouse-specific molecular probes, techniques such as chronic social defeat, ICSS, immunohistochemistry and RNAScope® *in situ* hybridization that are described here in rats, can be easily carried out in mice. This would allow us to conduct further experiments (such as those described in Sections 3.1.3, 3.3, 3.6 and 3.8.1), using transgenic mice such as *Vglut3-Cre*, *Pet1-Cre*, *Tph2-Cre*, *Gad1/2-Cre*, or their combinations, or a combination of Cre-based and Flp-based transgenic lines. Of the above transgenics, only *Tph2-Cre* and *Gad1-Cre* are currently available in rats.

3.1.2 Measurement of anhedonia and stress

As mentioned in Chapter 1, anhedonia can be measured using different tests. The ICSS procedure, which is used in this study, is a highly robust and specific measure of reward-seeking behavior. However, ICSS testing involves a long and complex protocol sometimes spanning 3 to 4 months, including anesthesia, surgery, recovery, multiple steps of behavioral training and baselining. This makes repeating experiments under various manipulations or larger sample sizes prohibitively time-consuming. Alternatives such as sucrose preference or social interaction are widely used but either lack reproducibility or specificity. However, they are much easier and quicker to conduct; and when correlated with anxiety measures such as open field test, elevated plus maze, light-dark maze and biochemical measures of stress like interleukin levels or cortisol levels, their predictive validity is improved. Therefore, they may be considered as alternative approaches to measuring susceptibility and resilience to anhedonia. Examining the correlations between the outcomes of each of these tests would in itself be informative. However, ICSS is still the most reliable and accurate single measure of anhedonia and should be replaced by other tests only after they are cross-validated with ICSS.

3.1.3 Evaluation of serotonergic expression

This study used an antibody to TPH2 to identify serotonergic neurons using immunohistochemistry, the rationale being that TPH2 is the only biosynthetic enzyme for serotonin in the central nervous system and therefore exclusively marks serotonergic neurons. At the mRNA level, *Pet1* was also investigated as it is a serotonergic neuron-specific transcription factor. In future experiments, PET1 can be investigated at the protein level using an antibody. Antibodies or mRNA probes to other markers such as serotonin transporter (SERT) or serotonin

itself can also be used. Validation with additional markers will categorically rule out the highly unlikely possibility that the results obtained in the current study were due to anti-TPH2 antibody-specific effects, thereby increasing confidence in the findings.

The accurate counting of immunostained neurons in brightfield or confocal micrographs is key to testing the hypothesis of neurotransmitter plasticity. In this study, accuracy was ensured by an experienced scorer performing exhaustive (non-stereological) counting of carefully chosen and delimited tissue sections; and analyzing section-averaged cell numbers to account for inter-section differences. Techniques to further improve the accuracy and reliability of cell counts will contribute to increasing the confidence in the results. One simple, yet powerful technique to improve accuracy and reliability is to have multiple blinded scorers of cell numbers and ensure inter-rater reliability above a pre-determined minimum before analysis, interpretation and communication of the counts.

Since the TPH2 antibody stains neuronal fibers and varicosities which can be mistaken for sectioned cell-bodies, and the DRv has a high packing density of TPH2+ neurons, estimation of cell number from images of TPH2-immunostained sections can be tricky. This can be addressed by performing the experiments in transgenic mice (or rats) expressing a nuclear-localized GFP or other reporter protein in serotonergic neurons. This can be accomplished by using the progeny of a *Tph2-Cre* mouse crossed to a mouse expressing a pan-neuronal floxed reporter with a nuclear localization sequence. However, this transgenic approach has the limitation that it includes the overhead of continuously breeding and rearing transgenics to generate the subjects for each experiment.

3.1.4 Sex differences

The current study used only male rats. To find out whether stress-induced anhedonia in females involves similar behavioral and molecular changes, it is important to perform these experiments in them as well. The only difficulty lies in the stress protocol – female social defeat is harder to achieve and less reliable. It can usually be performed only with lactating or pregnant female aggressors. This provides a very narrow window to use each aggressor, thereby demanding a larger colony of aggressors (with significant overhead costs) to have enough aggressors available to stress a full cohort of female subjects daily for 21 consecutive days, while also ensuring adequate rest to each aggressor (to prevent their fatigue). Once this hurdle is overcome either by developing a reliable and efficient female social defeat protocol or by using alternative stressors with comparable strength and validity, subsequent protocols like ICSS and histological studies are easily conducted in females. This will provide critical insights into sex differences (or lack thereof) which have major translational implications.

The rest of this chapter is organized as a series of questions that arise from the current study, each with a brief description of the experimental approach that could be used to address it.

3.2 What is the timeline of neurotransmitter plasticity following chronic social defeat?

The following four questions relate to the temporal progression of neurotransmitter plasticity and its regulation by DREADD-based activity manipulation.

3.2.1 At what timepoint in the social defeat paradigm does neurotransmitter plasticity begin to occur?

In the current study, a 3-week chronic social defeat protocol was followed and neurotransmitter expression was assessed in brain tissue that was fixed and collected at the end of the 21st day. To know the minimum number of days of stress required to induce neurotransmitter plasticity, rats can be sacrificed after 3, 10 or 14 days of social defeat for example, and assessed for the extent of neurotransmitter plasticity in the DRv. Since susceptibility/resilience manifest after the 11th day (Fig. 2.1), it is expected that plasticity arises after approximately 10 days which is consistent with timeline of neurotransmitter plasticity observed in other instances[114]. It would provide additional insight if resilient animals show a temporary increase in DRv serotonergic neurons prior to 10 days as a result of sub-chronic social defeat.

3.2.2 How long does the neurotransmitter phenotype in susceptible animals last after cessation of stress?

Der-Avakian et al.[38] subjected rats to social defeat and tested their reward (ICSS) behavior for 3 weeks post cessation of stress. Animals that were categorized as 'susceptible' or 'resilient' after 21 days of stress remained in the same categories even after 3 weeks of rest from social stress. It will be interesting to see if the increase in TPH2 and decrease in VGLUT3 in susceptible animals persist, like the reward thresholds, for 3 or even 6 weeks after cessation of stress. Persistence would lend further support to the conclusion that TPH2 plasticity is linked to anhedonic state, and VGLUT3 plasticity to stress. Return of neurotransmitter expression to control levels would indicate that neurotransmitter plasticity is driven by stress but is not required to maintain the acquired anhedonic state.

3.2.3 Does change in neuronal activity precede neurotransmitter plasticity and in which neurons does this occur?

As seen in Fig. 2.5, there are changes in activity levels of non-serotonergic neurons in the DRv following chronic stress. Activity changes are known to drive neurotransmitter plasticity[110,112,182], but the current study evaluated neuronal activity *after* neurotransmitter plasticity had already occurred. The manipulation of neuronal activity by CeA altered DRv neurotransmitter expression (Fig. 2.7) which suggests that stress-induced neurotransmitter plasticity in the DRv is activity-dependent. Evaluating cFos expression in serotonergic and non-serotonergic DRv neurons prior to development of susceptibility/resilience (based on results of experiments discussed in Section 3.2.1) would provide stronger evidence to support the idea that stress-induced activity changes in the DRv drive neurotransmitter plasticity leading to susceptibility.

3.2.4 Can susceptibility be fully reversed with a longer period of DREADD manipulation?

In the current study, DREADD treatment co-terminated with stress and this resulted in susceptible and resilient rats having statistically similar reward thresholds, but susceptible rats still had significant threshold elevations relative to controls (Fig. 2.6). It would be useful to test if prolonging DREADD manipulation after cessation of stress, completely reverses anhedonia in the susceptible rats and makes them resilient i.e. indistinguishable from controls. A related but subtly different modification of this approach would be to begin DREADD stimulation after cessation of stress; i.e. DREADD treatment would not overlap with stress but instead follow it. In humans, this would roughly translate to an attempt to treat or cure pre-existing stress-induced anhedonia after

the cessation of the stressor, as opposed to pre-emptive coping or preventive treatment, as has been attempted in this study (Fig. 2.6).

3.3 Are there additional reserve pools that gain TPH2+ phenotype?

VGLUT3+ neurons have been identified as a reserve pool in the DRv for gain of TPH2 in susceptible rats, while nitrergic and dopaminergic neurons have been eliminated as candidate reserve pools (Fig. 2.3). The large GABA-ergic population of neurons in the DRv[175] has not been tested as a potential reserve pool due to the unavailability of antibodies to GABA-ergic neurons that clearly stain cell bodies to enable cell counting. However, this challenge can be overcome using *Gad1/2-Cre* mice (or rats) and the approach described in Section 3.1.3.

3.4 Which other inputs to the DRv can be manipulated using DREADDs to induce susceptibility or resilience?

The following three questions relate to the inputs whose activity drives neurotransmitter plasticity in the DRv.

3.4.1 Which of the inputs to the DRv differ, between susceptible and resilient individuals, in their level of excitation following chronic stress?

The DRv and CeA are components of a larger circuitry that processes stress and reward. Other regions that project to the DR[96], like the prefrontal cortex, bed nucleus of stria terminalis, lateral habenula, lateral hypothalamus and other amygdalar nuclei, which are involved in stress processing, may also be involved in determining susceptibility and resilience. To test whether any of these regions plays a role, cFos immunostaining can be performed with comparisons of number of cFos+ neurons between control, susceptible and resilient animals. Regions that differ in their

level of excitation (as measured by cFos expression) between susceptible and resilient animals would be strong candidates to further test for their contribution to susceptibility and resilience, as discussed in the next two sections.

3.4.2 Does acute manipulation of these inputs cause changes in DRv activity?

Once candidate brain regions have been identified as described in Section 3.4.1, each of them can be manipulated acutely *in vivo* with simultaneous measurement of neuronal activity in the DR. The acute manipulation can be either via an implanted optogenetic fiber in an animal (mouse or rat) expressing channelrhodopsin or halorhodopsin in the region of interest; or via microinfusion of a DREADD agonist into the region of interest expressing excitatory or inhibitory DREADD receptors (chemogenetics), or via direct current stimulation of the region of interest. DR activity can be monitored either by electrophysiological recording (local field potentials or single-unit or multi-unit recording), or by fiber photometry in an animal expressing a genetically encoded calcium sensor and implanted with an optic probe in the DR.

3.4.3 Does chronic manipulation of these inputs cause changes in behavioral susceptibility/resilience and DRv neurotransmitter expression?

After confirming the effects of acute manipulation, as described in Section 3.4.2, chronic manipulation with DREADDs can be performed with simultaneous behavioral (ICSS) measurements followed by examination of DRv neurotransmitter expression. Similar to the manipulation of the CeA described in Chapter 2, this will reveal the effects of manipulating each relevant brain region on susceptibility/resilience to chronic stress-induced anhedonia and neurotransmitter plasticity in the DRv.

3.5 Does inhibition of CRH+ neurons in the CeA promote susceptibility and increased TPH2 expression?

The current study limited itself to hM3Dq-mediated activation of CeA CRH+ neurons, which led to the amelioration of anhedonia in susceptible rats and prevented stress-induced neurotransmitter plasticity in the DRv. However, whether the silencing of CeA CRH+ neurons will promote behavioral susceptibility and neurotransmitter plasticity is a question that remains to be answered. Similar to the DREADDs-based activation experiment described in Chapter 2, a hM4Di-mediated inhibition of CeA CRH+ neurons can be carried out to test the hypothesis that the activity of CeA CRH+ neurons is necessary to induce resilience and suppress DRv neurotransmitter plasticity.

3.6 Are the effects of manipulating CeA CRH+ neurons mediated by the direct CeA-DR projection?

In this study, AAV virus encoding Cre-dependent hM3Dq (excitatory DREADD) receptor was injected into the central amygdala as described in Section 2.4.3, resulting in the transfection of all CRH+ neurons in the CeA, irrespective of their projection targets. The effects of activating those neurons (Fig. 2.6, 2.7) are therefore not specific to the CeA-DR projection. Projection specificity (or its absence) can be demonstrated by a more involved strategy: transfecting the DRv of *Crh-Cre* rats (or mice) with a Flp-dependent retro-AAV virus encoding the DREADDs receptor, while also transfecting the CeA with a Cre-dependent Flp recombinase. This will lead to Flp expression in all CRH+ neurons of the CeA but only those that target the DR will also be transfected (retrogradely) with the Flp-dependent receptor. If the previously observed effects are also reproduced with this new experimental strategy, it would indicate that the manipulation of the

CRH+ CeA-to-DR projection is *sufficient* to produce the behavioral and molecular changes. However, if the previously observed effects are lost with the new experimental strategy, it would indicate that an indirect pathway beginning with the CeA CRH+ neurons, regulates DRv neurotransmitter plasticity and behavior.

3.7 What are the physiological effects of stress-induced neurotransmitter plasticity in the target areas of the switching population?

The current study shows the upregulation of TPH2, the biosynthetic enzyme for serotonin, in the DRv in susceptible rats. Whether this results in a corresponding increase in serotonin release at the synapses in the target regions is yet to be tested. The chief difficulty is in identifying the targets of *newly* serotonergic neurons. Based on findings (Fig. 2.3) that the plasticity occurs in a glutamatergic reserve pool and involves VGLUT3+ TPH2+ co-expressing neurons, the target regions of these dual serotonergic and glutamatergic neurons can be examined. Previous studies show that multiple brain regions are innervated by these dual neurotransmitter-expressing neurons[133,220,221]. Of these, the VTA and the amygdala are known for their role in the processing of reward and emotion. *In vivo* microdialysis studies in control, susceptible and resilient rats could be performed to measure extracellular 5-HT levels in target regions such as the VTA or amygdala. This can then be followed by electrophysiological recordings or fiber photometry measurements (in a fresh cohort of rats) in the target areas to determine differences in neuronal activity in these regions across experimental groups.

3.8 Cause or consequence?

As shown in Fig. 2.7, there is a positive correlation between reward thresholds and the number of DRv TPH2+ neurons. This raises the intriguing question of which one drives the other.

In other words, is neurotransmitter plasticity necessary and/or sufficient for susceptibility to stress-induced anhedonia?

3.8.1 Does blocking gain of TPH2 in stressed animals block the susceptible phenotype?

If neurotransmitter plasticity in the DRv is *necessary* for susceptibility, then blocking it in the presence of chronic stress should prevent anhedonia. An shRNA-mediated silencing of TPH2 in the DRv could be done to block TPH2 gain in stressed animals, followed by behavioral testing through a 21-day social defeat paradigm. A caveat with this approach is that ideally only the *newly* TPH2+ neurons should be silenced and not neurons that were already TPH2+, to ensure minimal off-target effects from disruption of the brain's serotonergic system, and also to specifically test the hypothesis that TPH2 *gain* (and not TPH2 itself) is necessary for susceptibility. In order to ensure this, it would be necessary to target the reserve pool specifically and completely. A possible approach to achieve this would be to conduct this experiment in mice that express Cre in *all* identified reserve pools; i.e. if experiments described in Section 3.3 reveal that GABAergic neurons also form a reserve pool, then a mouse that is *Vglut3-Cre* as well as *Gad1/2-Cre* (progeny of the appropriate cross) should be transfected with Cre-dependent shRNA against TPH2. While this approach limits the shRNA expression to the reserve pool, it may still lead to reduction of TPH2 levels to below control/resilient levels, as neurons that would ordinarily be VGLUT3+ TPH2+ or GAD1/2+ TPH2+ in control/resilient conditions would also lose TPH2. With the help of weak promoters controlling the shRNA expression, this disadvantage can potentially be minimized.

3.8.2 Does overexpression of TPH2 in the DRv in an animal cause susceptibility?

If gain of TPH2 within the reserve pool is *sufficient* for behavioral susceptibility then overexpression of TPH2 in the DRv, even in the absence of stress, will lead to anhedonia (increased reward thresholds). This can be tested by overexpressing TPH2 in the DRv in unstressed rats and measuring their ICSS thresholds. Since ICSS thresholds are useful only when measured as relative changes to baseline, the overexpression would have to be inducible and induced after ICSS baselines have stabilized. The TPH2 overexpression can be conducted within the reserve pool as described in the previous section. A caveat with TPH2 overexpression is that it might not be sufficient in itself to lend 'serotonergicity', which as mentioned in Section 1.5, involves the expression of other serotonin-related genes in addition to TPH2. Overexpressing PET1 instead may help to overcome this challenge.

Taken together, if the experiments described in Sections 3.8.1 and 3.8.2 prove that neurotransmitter plasticity is necessary and sufficient for behavioral susceptibility, it will firmly establish that DRv neurotransmitter plasticity *causes* stress-induced anhedonia. Otherwise, if stress-induced anhedonia occurs even when neurotransmitter plasticity is blocked or if it fails to occur even when the number of DRv TPH2+ neurons is artificially increased in unstressed animals, then it would suggest that neurotransmitter plasticity in the DRv occurs *in response to* or *as a consequence of* anhedonia.

In conclusion, the study described in Chapter 2 indicates that an activity-dependent neurotransmitter plasticity mechanism in the DRv characterizes susceptibility/resilience to stress-induced anhedonia. Questions relating to causality, to the exact temporal progression of this plasticity, the details of its regulation by the central amygdala and the relation of neurotransmitter

plasticity to the input and output nuclei of the DRv, are further avenues to be explored. It is hoped that further studies will unravel these details and provide valuable pre-clinical insights to scientists and medical practitioners formulating new treatments for depression and other mental disorders.

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